Haemolymph and gut microbiomes of the ornate spiny lobster *Panulirus ornatus*

By

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Declarations

Declaration of originality

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Statement of Co-Authorship

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Abstract

The reliance on wild stocks of ornate spiny lobster (*Panulirus ornatus*) for aquaculture and fisheries production will be reduced with the recent development of hatchery technology. The commercialisation of *P. ornatus* closed life cycle aquaculture production is nearing completion thus demanding greater understanding of various aspects of lobster health. In these early stages of sector development, each animal produced is valuable from both an economic and research standpoint, and so nonlethal sampling methods using haemolymph (blood) are preferred in health monitoring programs. Bacterial communities are indigenous to the haemolymph, a condition termed asymptomatic bacteraemia. However, dysbiosis of bacterial communities within the haemolymph can lead to invasion and proliferation of pathogens, causing disease. Limited information exists for the bacterial composition and diversity associated with the haemolymph of healthy and diseased states of *P. ornatus*. This knowledge gap can be filled using next generation DNA sequencing, a technical platform used to answer similar questions in the Human Microbiome Project. The primary aim of this study was to characterise haemolymph bacterial communities of healthy *P. ornatus* juveniles, deduce their potential functions and compare them with the gut microbiome.

Next generation sequencing of bacterial 16S rRNA genes showed that the core haemolymph microbiome of juvenile *P. ornatus* consisted of phyla Proteobacteria and Bacteroidetes. Culture-independent analysis of bacterial communities captured a higher bacterial diversity comprising 13 phyla when compared to culture-dependent methods (2 phyla), yet members of the family *Rhodobacteraceae* were strongly represented in both analyses. Haemolymph bacterial loads of control

lobsters ranged from 0 to 3.1×10^3 CFU mL^{-1} (culturable bacteria) and from 55 to 2.8×10^3 cell equivalent mL^{-1} (*rpoB* gene). The difference in culture-dependent and -independent analyses reinforces the limited resolving power and bias of the former method, a likely variable in the reporting of haemolymph-associated bacteria in earlier studies on lobsters. The observed prevalence and diversity of certain groups of bacteria in the haemolymph was suggestive of a number of positive functional roles including antimicrobial production and contribution to host nutrition. Juveniles thermally challenged for 6 d at 34 °C, which is 6 °C above the standard culture temperature of 28 °C, were affected in terms of survival, total granulocyte counts, bacterial diversity, bacterial load and functional potential of the haemolymph bacterial community. This study advocates nonlethal sampling and closer examination of the haemolymph microbiome for use in health monitoring programs of cultured spiny lobsters.

The gut microbiome of juvenile *P. ornatus* across region and developmental stage was characterised and screened for similarities with the haemolymph microbiome. Next generation DNA sequencing showed the core gut microbiome consisted of phyla Tenericutes and Proteobacteria, with communities dominated by families *Pseudoalteromonadaceae* and *Vibrionaceae*. Gut region shaped the bacterial communities of 13 month post-emergence lobsters, where the hindgut community was more diverse and compositionally dissimilar to the foregut and midgut bacteria. The bacterial composition of the hindgut was influenced by developmental stage as there were more similarities among younger juveniles (6 - 7 d and 52 d post-emergence) compared to 13 month post-emergence lobsters. Bacteria identified as common to both the gut and haemolymph microbiomes included *Flavobacteriaceae*, *Saprospiraceae*, *Rhodobacteraceae*, *Moraxellaceae*, *Pseudoalteromonadaceae* and *Vibrionaceae*, suggestive of a transmission mechanism. This study was the first to comprehensively explore gut microbiomes of spiny lobster juveniles and to demonstrate that the bacteria present were influenced by gut region and developmental stage.

Bdellovibrio and like organisms (BALOs) are obligate predators of other bacteria and were discovered for the first time in the present work residing in the haemolymph and gut of juvenile *P. ornatus*. BALOs have strong potential as biocontrol agents and alternatives to antibiotics in aquaculture, warranting further investigation into their roles in spiny lobster hosts. A combination of co-culture assays (agar and broth) and transmission electron microscopy was used to show a BALO strain (*Halobacteriovorax* sp.) isolated from sea water preyed upon a haemolymph-derived prey bacterium *Vibrio* sp. When *Halobacteriovorax* sp. was injected into the circulatory system of lobsters, there was no mortality and no change in immune parameter (percentage of granulocytes) or haemolymph microbiome after 3 d. However, lobsters injected with both prey and predator showed significant differences compared to other treatments (PBS control, prey only, predator only) in haemolymph bacterial community composition and reductions in plasma DNA concentration (bacterial load) after 2 d. The results indicate that BALOs are not pathogenic to juvenile *P. ornatus* and may assist the host in controlling bacterial population growth in the haemolymph.

This study provides an extensive baseline characterisation of haemolymph and gut microbiomes of healthy cultured *P. ornatus* juveniles. Future comparisons with microbiomes of diseased individuals and those subject to suboptimal environmental conditions will advance the development of health management strategies for *P. ornatus*.

Chapter 1 General introduction

1.1 Spiny lobster taxonomy and biology

Ornate spiny lobsters *Panulirus ornatus* belong to the phylum Arthropoda, subphylum Crustacea, class Malacostraca, order Decapoda and family Palinuridae. These clawless marine species are different from families of true lobsters (eg. Nephropidae, Astacidae) which are defined by the presence of chelae (claws) as modified forms or extensions of pereiopods (walking legs). Other crustaceans that share the same order include crabs and shrimps (prawns).

The life cycle of *P. ornatus* involves incremental growth through a series of moults and metamorphic changes (Figure 1-1). Following hatching from eggs, larvae (termed phyllosomas) are released into pelagic environments where they pass through up to 11 distinct morphological stages over a 3 – 8 month period (Smith et al., 2009) and exhibit diurnal vertical migration (DVM) behaviours for targeting prey (Booth and Phillips, 1994). Final stage phyllosomas metamorphose into pueruli, which is a short non-feeding stage and where the pueruli are capable of lateral propulsion allowing them to migrate to coastal onshore environments. After benthic settlement, pueruli emerge into early juveniles, recommence feeding and become pigmented (Butler IV and Herrnkind, 1991). Juvenile developmental stages have been termed days or months ‘post-emergence’ (dpe or mpe) in this thesis. Growth of juveniles continues through numerous moults until reaching an adult stage.

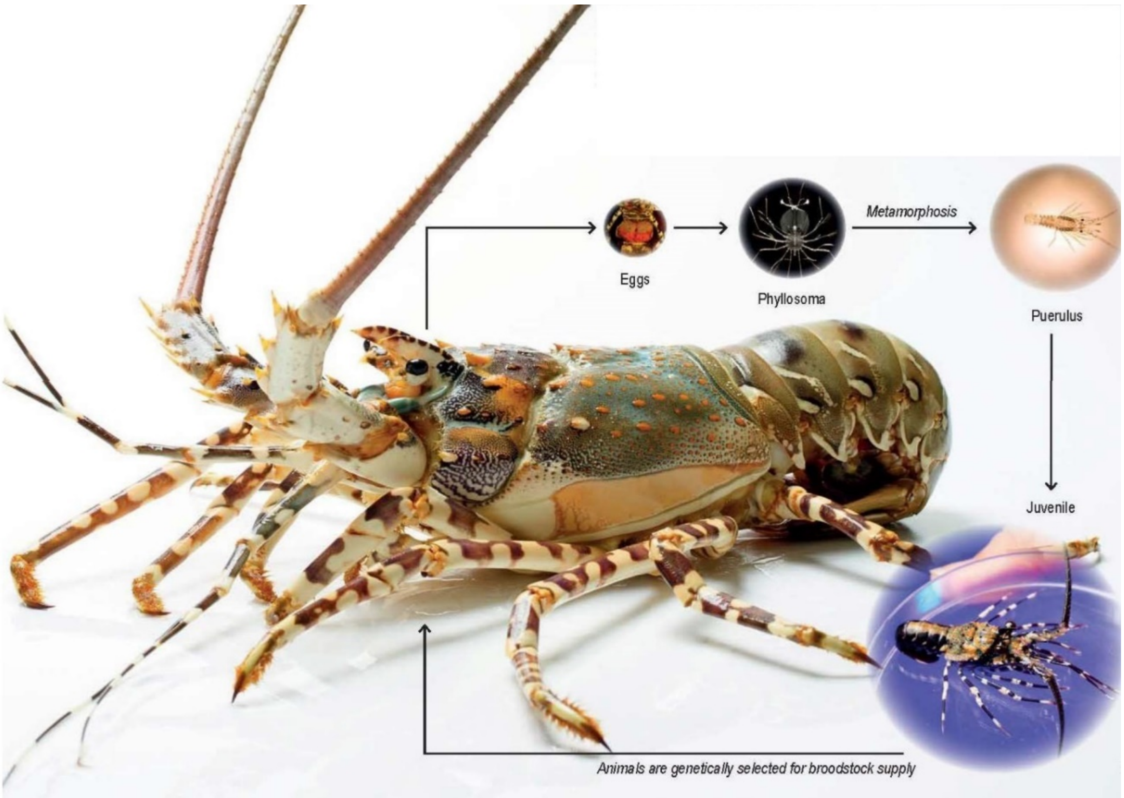

Figure 1-1. Life cycle of *Panulirus ornatus* (Image: IMAS).

1.2 Spiny lobster aquaculture

Panulirus ornatus is extensively distributed over the Indo-West Pacific region where it supports wild-caught fisheries and grow-out aquaculture operations. Both these industries are reliant on wild stocks which are generally in decline (Jeffs, 2010). Therefore, *P. ornatus* has been targeted for closed life cycle aquaculture to reduce the impact on wild stocks. Additionally, *P. ornatus* is an attractive aquaculture species because of its fast growth rate and high market price in Asia (Jeffs, 2010). However, the major challenges to the development of a viable closed life cycle aquaculture sector for ornate spiny lobster lie within the management of the long and complex larval phase, disease susceptibility, and cannibalism (Jeffs, 2010). Following 18 years of research on nutrition, water treatment and mass larval rearing systems, UTAS-Nexus Aquasciences Pty. Ltd. (UNA) have managed to breed and grow *P. ornatus* from egg to adult in commercially supported numbers. The world's first commercial hatchery is now currently under construction in Tasmania.

1.2.1 Lobster health

Aquaculture particularly at high densities can affect lobster health if poorly managed. Disease can occur when lobster immunity is compromised and / or when the farming environment favours the proliferation of pathogens. Although non-infectious health issues of lobsters have been reported, infectious diseases are more prevalent and damaging to animal production and profitability (Shields, 2011). Bacteria are one of the most common lobster pathogens encountered in experimental culture, holding systems, and grow-out cages. Well known examples of bacteria causing systemic diseases in lobsters are *Rickettsia*-like bacteria (Nunan et al., 2010), *Vibrio* spp. and *Aerococcus viridans* (Shields, 2011). The standard biological tissue used for the diagnosis of systemic diseases is haemolymph.

1.2.2 Potential disease management strategies of cultured lobsters

As bacteria pose a risk to cultured lobsters, disease management strategies are required to ensure animal health and production targets are met. Strategies for preventing bacterial diseases include seawater ozonation (Ritar et al., 2006), probiotics (Zhuo and Wang, 2012; Llewellyn et al., 2014), immunostimulation (Smith et al., 2003) and phytogenics (Menanteau-Ledouble et al., 2015). Following bacterial disease outbreaks in cultured lobsters, curative treatment options include antibiotics, quorum sensing disruption, bacteriophages (Defoirdt et al., 2011) and predatory bacteria (Damron and Barbier, 2013). Although antibiotics are perceived as a rapid and effective method for treating bacterial diseases, there is now increasing concern for the over- and indiscriminate use of these compounds giving rise to antibiotic resistance, rendering bacteria invulnerable to certain antibiotics (Damron and Barbier, 2013).

1.3 Lobster circulatory system

Lobsters have an open circulatory system (McLaughlin, 1983) in which haemolymph, comprising plasma and suspended haemocytes (circulating cells), flows freely in the haemocoelic cavity and perfuses all tissues directly (Martin and Hose, 1995; Wirkner et al., 2013). There are three types of haemocytes in lobsters including hyalinocytes, semigranulocytes and granulocytes (Evans et al., 2002). The circulation of haemolymph is influenced by pumping of the heart, body movements, and muscular and gut contractions (McLaughlin, 1983). The general functions of the circulatory system are homeostasis, transportation (oxygen is carried by haemocyanin), hydraulics and protection (Wirkner et al., 2013). Protection consists of both cellular and humoral processes, performing basic functions including coagulation, phagocytosis, degranulation, encapsulation and haemocytosis (Shields et al., 2006).

1.4 Bacteria associated with lobsters

1.4.1 Bacteria in the haemolymph

Although haemolymph is the biological tissue of choice for diagnostics, particularly for systemic diseases, the haemolymph of healthy lobsters has been reported to contain bacteria. Without showing clinical signs of disease, this condition is called asymptomatic bacteraemia. Previous studies have reported 5 to 100 % prevalence with 1 to 16 species of bacteria in the haemolymph of apparently healthy wild and cultured lobsters (Table 1-1). Using culture methods, the levels of bacteria formerly reported ranged from below detection to 623 CFU mL^{-1} of haemolymph (Mary Leema et al., 2010; Sang and Fotedar, 2010).

Early work by Stewart (1984) suggested that non-pathogenic bacterial populations circulating within the haemolymph are transient and are likely to be a result of trauma. This was supported by Martin et al. (1993) and Martin et al. (2000) who stated that crustaceans remove circulating foreign particles including bacteria from the haemolymph rapidly. However, emerging research has indicated that bacterial populations in the haemolymph are tightly regulated by the host. For example, Fagutao et al. (2009) indicated that the proliferation of bacteria in the haemolymph is controlled by the prophenoloxidase system in healthy kuruma shrimp, *Marsupenaeus japonicus*. Furthermore, some of the bacterial groups consistently recovered from the haemolymph of crustaceans may have positive roles in host health. For instance, the majority of haemolymph samples taken from wild American lobster *Homarus americanus* harboured *Sediminibacterium* sp. and *Ralstonia* sp., which are closely related to symbionts of insects (Quinn et al., 2013).

Table 1-1. Reports of bacteria in the haemolymph of lobsters. NA: not available.

Host	**Geographic location**	**Host age group, condition**	**Identification and/or quantification method**	**Prevalence of bacteria in the haemolymph % (sample size)**	**Bacterial load**	**Bacterial diversity**	**Reference**
Ornate spiny lobster *Panulirus ornatus*	Queensland, Australia	Juvenile, acclimated from wild	Biochemical	43 (30)	NA	• *Pseudomonas* sp. • *Vibrio* sp. • *Aeromonas* sp. • *Micrococcus* sp.	Evans et al. (2003)
	Nha Trang bay, Vietnam	Juvenile, acclimated from wild	Culture	NA (6)	283 ± 37 - 623 ± 66 CFU mL^{-1}	• NA	Sang and Fotedar (2010)
Western rock lobster *Panulirus cygnus*	Western Australia	Almost mature, wild	Biochemical	15 (13)	NA	• *V. alginolyticus*	Evans et al. (1996)
	Western Australia	Adult, acclimated from wild	Biochemical	100 (48)	NA	• *Rhodococcus maris*	Evans et al. (2002)
	Western Australia	Low and high stress	Culture	Low stress (20) High stress (16)	Low stress- max 2.5×10^2 CFU mL^{-1} High stress- max $>3\times10^3$ CFU mL^{-1}	• NA	Evans et al. (2002)
Scalloped spiny lobster *Panulirus homarus*	Persian Gulf, Iran	Wild	Biochemical and molecular	10 (60)	NA	• *V. vulnificus, V. harveyi, V. mimicus, V. alginolyticus*	Raissy et al. (2011)
	India	Juvenile, shell disease	Culture	75 (Diseased-30, Healthy-10)	Diseased- 26.6-51.8 CFU mL^{-1} Healthy- below detection	• *Vibrio sp.*	Mary Leema et al. (2010)

Host	Geographic location	Host age group, condition	Identification and/or quantification method	Prevalence of bacteria in the haemolymph % (sample size)	Bacterial load	Bacterial diversity	Reference
Southern rock lobster *Jasus edwardsii*	Tasmania, Australia	Juvenile, wild	Biochemical	13 (46)	NA	• *V. splendidus* I, *V. navarrensis*	Handlinger et al. (2006)
		Juvenile, farmed on-shore & sea-cage	Biochemical	5 (41)	NA	• *V. splendidus* I	
American lobster *Homarus americanus*	Long Island Sound, USA	Wild	Biochemical	West - 88 (60) East – 51 (168) Off-shore – 15 (33)	NA	• *V. fluvialis* • *Pseudomonas putida* • *Stenotrophomonas* (*Pseudomonas*) *maltophilia* • *Brevundimonas* (*Pseudomonas*) spp. • *Delftia* (*Pseudomonas*) *acidovorans* • *Klebsiella pneumonia* • *Chryseobacterium* spp.-like • *Hafnia alvei* • *Enterococcus faecalis*, resembling *Enterococcus* • *Corynebacterium*-like • Coagulase-negative *Staphylococcus* spp., *S. equorum*, *S. warnerii, S. epidermis, S. xylosus* • Alpha-haemolytic *Streptococcus*-like	Bartlett et al. (2008)

Host	**Geographic location**	**Host age group, condition**	**Identification and/or quantification method**	**Prevalence of bacteria in the haemolymph % (sample size)**	**Bacterial load**	**Bacterial diversity**	**Reference**
American lobster *Homarus americanus*	USA and Canada	Wild, epizootic shell disease free	Nested-PCR and DGGE	100 (13)	NA	• *Flavobacteria* sp. • *Weissella paramesenteroides* • *Novosphingobium* sp. • *Ralstonia* sp. • *Delftia acidovorans* • *Pelomonas aquatic* • Uncultured Alphaproteobacteria • Uncultured *Hyphomicrobium* sp. • Uncultured *Sphingobacteriales* • Uncultured Deltaproteobacteria • *Stenotrophomonas maltophilia*	Quinn et al. (2013)
	Canada	Wild, survey	Culture (selective medium for *Gaffkya homari*)	20 (2035)	NA	• *Brevibacterium* • *Achromobacter superficialis* • *Pseudomonas* • *Micrococcus*	Cornick and Stewart (1966)

1.4.2 Factors affecting bacteria in the haemolymph

Bacterial load in the haemolymph can be affected by host stress. Stress responses are triggered by changes in the environment including water quality parameters, physical factors, behavioural interactions and nutrient availability (Evans, 1999). Environmental stressors such as increased water temperature and prolonged air exposure are positively correlated with bacterial load in the haemolymph of red swamp crawfish *Procambarus clarkii* (Scott and Thune, 1986) and western rock lobster *P. cygnus* (Fotedar et al., 2001). Increased culture duration and developmental stage have also been linked to bacterial proliferation in the haemolymph of noble crayfish *Astacus astacus* (Madetoja and Jussila, 1996) and black tiger shrimp *Penaeus monodon* (Ruangpan et al., 1994). Changes in the levels of bacteria in the haemolymph are often related to disturbances in haematological parameters. For instance, shell-diseased scalloped spiny lobster *P. homarus* (Mary Leema et al., 2010) and air-exposed *P. cygnus* (Fotedar et al., 2001) with increased bacteraemia levels showed increased clotting time and decreased total haemocyte count and serum protein.

The change of state of the cuticle during the moult cycle can increase the risk of bacterial infection. When the old cuticle detaches during the premoult phase, bacteria can penetrate into the body through the new cuticle (Le Moullac et al., 1997). In contrast, during intermoult the cuticle is sclerotinised and bacteria can only enter the cuticle when there is an injury (Le Moullac et al., 1997). The crustacean immune response to bacterial invasion also changes during the moult cycle. During pre- and postmoult, crustaceans can exhibit reduced phenoloxidase activity, phagocytic activity and clearance efficiency against pathogens (Cheng et al., 2003).

For example, mortality was highest in the giant freshwater prawn *Macrobrachium rosenbergii* (Cheng et al., 2003) and blue shrimp *Penaeus stylirostris* (Le Moullac et al., 1997) injected with pathogenic bacteria during premoult and during postmoult in the Pacific white shrimp *Litopenaeus vannamei* (Liu et al., 2004).

1.4.3 Bacterial transmission

The presence of bacteria in the haemolymph raises the question of portals of entry. Bacteria in the environment such as water and feed may enter the circulatory system via injured integument, gills (osmoregulation) and the digestive tract (Cornick and Stewart, 1966; Kesarcodi-Watson et al., 2008). The disease Gaffkaemia is an example whereby a bacterium (*Aerococcus viridans*) enters lobsters via damaged integument and proliferates in the hepatopancreas and heart before spreading to the haemolymph (Stewart and Arie, 1973). Increased bacterial loads in the haemolymph have also been associated with microbial conditions such as epizootic shell disease in *H*. *americanus* (Homerding et al., 2012) and shell disease in crab *Cancer pagurus* (Vogan and Rowley, 2002). However, Chistoserdov et al. (2005) and Quinn et al. (2013) have shown that bacterial assemblages associated with cuticular lesions are distinct from those found in the haemolymph of *H. americanus*. Therefore, bacterial load should be studied together with bacterial diversity to allow more accurate interpretations. Nonetheless, a comparison of bacterial compositions of the digestive tract and haemolymph may infer a transmission mechanism. Vertical transmission from mother to progeny is another possible source of haemolymphic bacteria (Fagutao et al., 2009).

1.4.4 Bacteria in the gut

The digestive tract of lobsters is divided into three main regions: foregut, midgut and hindgut. The foregut and hindgut is lined with chitin and is shed during moulting, but this does not occur in the midgut (Ceccaldi, 1989). The foregut consists of the mouth, oesophagus and stomach, where feed is brought to the mouth, trafficked through the short oesophageal duct and ground mechanically in the stomach (Perera and Simon, 2015). The ground food particles enter the hepatopancreas (midgut gland) to be digested enzymatically, allowing for absorption and storage of nutrients. The indigestible particles are removed of fluids before being coated with a peritrophic membrane in the midgut. The coated faecal pellet moves to the rectum and anus of the hindgut for excretion (Ceccaldi, 1989).

The digestive tract has constant exposure to feed and water that carry bacteria. Hence, healthy hosts including lobsters always have bacteria in their gut (Table 1-2). However, Meziti et al. (2012) found that the majority of gut bacteria in the Norway lobster *Nephrops norvegicus* were different from those in the water and diet. This may be due to host selection of resident bacteria for certain purposes in the gut environment (Rungrassamee et al., 2014). Therefore resident gut bacteria may have functional roles in the global health condition of lobsters (Immanuel et al., 2006), including digestion, growth and development, immune response, and promoting disease resistance by outcompeting invasive and pathogenic microorganisms (Harris, 1993; Broderick and Lemaitre, 2012; Minard et al., 2013).

Table 1-2. Bacteria in the gut of apparently healthy lobsters.

Lobster species	Gut bacteria	Reference
Scalloped spiny lobster *P. homarus*	• *Pseudomonas aeruginosa* • *Vibrio parahaemolyticus* • *Bacillus circulans* • *Escherichia coli* • *Photobacterium damselae* • *Flavobacterium columnare* • *Micrococcus luteus* • *Enterobacter aerogenes* • *Corynebacterium xerosis* • *Alcaligenes*	Immanuel et al. (2006)
Japanese spiny lobster *Panulirus japonicus*	• *Vibrio* • *Pseudomonas* • *Staphylococcus* • Coryneforms	Sugita et al. (1987)
Norway lobster *Nephrops norvegicus*	• *Photobacterium* sp. • *Colwellia* sp. • *Arcobacter* sp. • *Vibrio* sp. • *Phaeobacter* sp. • *Litoreibacter* sp. • *Pseudoalteromonas* sp. • *Defluviicoccus* sp. • *Candidatus* Hepatoplasma • *Shewanella* sp. • *Propionibacterium* sp. • *Marivita* sp. • Uncultured Bacteroidetes	Meziti et al. (2012)
American lobster *H. americanus*	• *Rahnella* spp. • *Aranicola* sp. • *Serratia* spp. • *Escherichia coli* • *Vibrio* spp. • *Pseudomonas aeruginosa* • *Halomonas* spp. • *Bacillus* spp. • *Psychroserpens burtonensis* • *Lutibacter litoralis* • *Cytophaga* sp. • *Flavobacterium* sp. • *Tenacibaculum maritimum* • *Polaribacter* spp.	Battison et al. (2008)

1.4.5 Methods to detect and quantify bacteria

Haemolymph, like many biomes, will contain both culturable and unculturable bacteria. Due to the possibility of low levels of bacteria present in the haemolymph, the ability to culture particularly rare and fastidious bacterial groups is being questioned when a low volume of haemolymph is extracted and plated. This could be the reason for bacteria not being recovered from lobster haemolymph in some culture-based studies. Although not very sensitive, culture methods are required to obtain viable bacteria. Pure cultures of live bacteria are often needed for identification, and subsequent *in vitro* and *in vivo* experiments, including experimental infection assays and probiotic studies (Foligne et al., 2007).

Molecular methods such as polymerase chain reaction (PCR) and quantitative PCR (qPCR) can detect both culturable and unculturable bacteria. More specifically, molecular methods can detect nucleic acids from live, dead, and viable but non-culturable (VBNC) bacteria (Horz et al., 2005). As part of bacterial nucleic acids, the 16S small subunit (SSU) rRNA (ribosomal RNA) gene is commonly used in the phylogenetic identification of bacteria (Klindworth et al., 2012). This housekeeping gene is highly conserved while containing hypervariable regions that allow identification of specific bacteria. However, accurate identification may be confounded when novel bacteria are recovered, 16S rRNA gene sequence databases contain limited and unverified sequence deposits, and when intraspecies variability is present (Clarridge, 2004; Janda and Abbott, 2007). Therefore, protein coding genes including the *rpoB* gene are increasingly being used for bacterial studies (Case et al., 2007). The copy number of the 16S rRNA gene varies (i.e. 1 – 14 copies) (Liu et al., 2012) between bacteria but is fixed (i.e. 1 copy) for the *rpoB* gene (Case et al., 2007). This approach may ease comparison between samples when high diversity is expected, such as in unexplored environments and animal microbiomes.

Universal bacterial primers (eg. 16S rRNA gene primers) are usually employed during sequencing for bacterial identification. Sanger sequencing can be used for identification of a bacterium in a pure culture. Newer next-generation sequencing (NGS) is more suitable for complex microbial communities such as in the haemolymph. NGS has expanded allowing sequencing of a specific gene or a whole genome. Although 16S rRNA gene has been thought to be present in almost all bacteria, a new branch of bacteria without that gene was discovered recently (Eng, 2016). Therefore, sequencing of the whole genome will lead to higher chances of the detection of all bacteria present in a sample. However, the challenges of whole-genome sequencing (metagenome) are potential contamination with host DNA and presence of numerous genes of unknown function or insufficient quality annotation (Petrosino et al., 2009).

1.4.6 Predatory bacteria associated with lobsters

Bdellovibrio and like organisms (BALOs) are a group of Gram negative bacteria consisting of families *Bdellovibrionaceae*, *Bacteriovoracaceae*, *Halobacteriovoraceae* and *Peredibacteraceae* (Rotem et al., 2014; Koval et al., 2015). BALOs share a unique behavioural trait by preying upon other Gram negative bacteria. Thus, BALOs act as a natural top-down control mechanism of bacterial communities. They have been reported in the environment (e.g. water, soil) (Amat and Torrella, 1989; Davidov et al., 2006; Wen et al., 2009; Chu and Zhu, 2010) and animals (e.g. gut, gill, shell) (Kelley and Williams, 1992; Pineiro et al., 2007; Cao et al., 2014b). The broad spectrum bacteriolytic effect of BALOs have enabled them to be used as biocontrol agents, conferring host protection against numerous aquatic animals pathogens (Cao et al., 2014a; Cao et al., 2014b; Cao et al., 2015). Two BALO members, *Bdellovibrio* and *Bacteriovorax*, were discovered in the haemolymph and hindgut of *P. ornatus* (Chapters 3 and 4). Their presence in the haemolymph of lobsters may suggest positive functional roles and warrants further investigation.

1.5 Aims

Most previous studies on Palinurid lobsters have relied on conventional microbiological techniques to characterise bacterial assemblages and these often exclude rare, nonculturable and nonviable groups. Therefore, little information is known about bacteria in the haemolymph of *P. ornatus* juveniles. The main aims of this thesis were to characterise bacterial diversity and load in the haemolymph of healthy *P. ornatus* and deduce their potential functions, and compare to descriptions of the gut microbiome alongside environmental and host factors. The haemolymph baseline information will be foundational to future diagnoses of diseased conditions.

Specifically, the four main aims of this thesis were:

- To identify bacteria in the haemolymph and gut of *P. ornatus* juveniles using a culture-dependent method;
- To identify and quantify bacteria in the haemolymph of *P. ornatus* juveniles and examine the effect of an environmental factor (increased temperature) using culture-dependent and culture-independent methods;

- To identify bacteria in the gut of *P. ornatus* juveniles and examine the effects of host factors (developmental stage and gut region) using a culture-independent method; and
- To examine the effect of *Bdellovibrio* and like organisms in the circulatory system of *P. ornatus*.

1.6 Notes on this thesis

Experimental chapters 3 to 5 in this thesis have been prepared or published in the following journals:

Chapter 3

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Chapter 4

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Chapter 5

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Hence, some content may be repeated particularly in the introduction and materials and methods sections.

Chapter 2 Culturable bacteria in the haemolymph and gut of the ornate spiny lobster *Panulirus ornatus*

2.1 Abstract

Previous studies on the presence of culturable bacteria in the circulatory system of lobsters have not been consistent. One of the hypotheses for the source of bacteria in the haemolymph is transmission from an injured digestive tract. This preliminary study aimed to characterise the culturable microbial diversity in the haemolymph of healthy cultured *P. ornatus* juveniles. In addition, bacteria in the haemolymph and digestive tract (hepatopancreas, midgut and hindgut) were compared between healthy lobsters and an abnormal individual. Samples were obtained from two 8 days post-emergence and eleven 11 months post-emergence healthy animals, and one abnormal individual. Strains isolated on Zobell marine agar were identified by Sanger sequencing of the 16S rRNA gene. Overall, the isolates from the haemolymph of healthy juveniles represented 10 bacterial genera particularly from families *Rhodobacteraceae* and *Flavobacteriaceae*. In contrast, five bacterial genera predominated by *Vibrionaceae* were cultured from the digestive tract. Interestingly, and unlike healthy lobsters, the abnormal lobster had *Vibrio* populations in the haemolymph. This study shows that the haemolymph of healthy *P. ornatus* is not sterile.

2.2 Introduction

Currently, the ornate spiny lobster *P. ornatus* available to consumers is derived from wild-caught fisheries and grow-out aquaculture industries. Grow-out operations involve the unsustainable practice of collecting seedstock (puerulus and early juveniles) from the wild. Eventually, the advancement of hatchery technology for closed life cycle production in recent decades (Bermudes and Ritar, 2008; Hall et al., 2013) will eliminate the dependence on wild stock and make it less vulnerable to overfishing and the effects of climate change.

Aquatic animals are constantly exposed to bacteria in the surrounding environment and microbes may be taken up during the process of feeding (Kesarcodi-Watson et al., 2008). Gut bacteria have functional roles in the health and growth of lobsters. In particular, gut compositions that include *Vibrio* and *Pseudomonas* spp. have frequently been isolated from the gut of healthy lobsters with putative roles in food digestion and production of antimicrobials (Sugita et al., 1987; Immanuel et al., 2006; Battison et al., 2008; Meziti et al., 2012). As the digestive tract is divided into three regions: foregut, midgut (including hepatopancreas) and hindgut, the bacteria found could differ according to the ecophysiology (eg. nutrients, structures, conditions) of each region (Harris, 1993). Furthermore, in healthy crustaceans organs such as the hepatopancreas bacterial populations are controlled by haemocytes (Martin et al., 1993). However, an injured digestive tract may allow bacteria to transfer to the haemolymph (Cornick and Stewart, 1966), where some may exhibit pathogenic effects.

Previous reports on the presence of bacteria in the circulatory system of lobsters have not been consistent. Haemolymph bacteria have been found in several healthy lobsters (Evans et al., 2002) but were reported absent in other routine health examinations (Shields, 2011). The prevalence of asymptomatic bacteraemia in previous studies on lobsters is likely to be associated with the culture-dependent microbiological methods. The type of culture medium, volume of haemolymph and incubation condition (eg. temperature, aerobic/anaerobic) can all influence the presence and diversity of culturable bacteria. Traditionally, bacterial isolates from plate cultures are identified using a series of biochemical tests. However, these tests are designed for the identification of common species whose phenotypical characteristics are well described. Hence, gene sequencing is preferred for the identification of bacteria from tissues and environmental samples. One of the most used genes is the 16S rRNA gene which is highly conserved and present in almost all bacteria (Janda and Abbott, 2007). The majority of previous studies have examined the prevalence of asymptomatic bacteraemia of wild or wild-acclimated lobsters. The main aim of this preliminary study was to characterise the microbial diversity in the haemolymph of healthy cultured *P. ornatus* juveniles. In addition, bacteria in the haemolymph and digestive tract were compared between healthy and abnormal lobsters. A culture library that can be used for future studies was constructed from the bacteria isolated from the animals.

2.3 Materials and methods

2.3.1 Juvenile lobster culture system

Panulirus ornatus were reared from embryos that hatched as larvae and metamorphosed into juveniles at the Institute for Marine and Antarctic Studies (IMAS), Hobart, Australia. Instar 1 juveniles (J1) were reared individually in divided 8 L containers supplied with flow through seawater (temperature 27.72 ± 0.04 °C; dissolved oxygen [DO] 105.30 ± 0.66%; pH 8.19 ± 0.01 and salinity 33.57 ± 0.15 ppt) at 6.0 exchanges h^{-1}. Animals were provided with cylindrical PVC tubes as hides and cultured in predominate darkness. J1s were fed fresh blue mussel (*Mytilus galloprovincialis*) mantle once daily and chopped commercial Kuruma prawn pellet (Higashimaru, Vital No. 12, http://www.k-higashimaru.co.jp/) twice daily. Two apparently healthy 8 days post-emergence (dpe) J1 (0.13 ± 0.02 g; intermoult) were sampled.

Eleven months post-emergence (mpe) juveniles were held communally in 600 L Y-Not fibreglass vessels supplied by flow through seawater (temperature 26.44 ± 0.08 °C; DO 100.01 ± 0.47%; pH 7.88 ± 0.01 and salinity 34.50 ± 0.12 ppt) at 0.36 exchanges h^{-1}. Animals were provided with Z-stack plastic hides and reared under 12 h light / 12 h dark photoperiod. Juveniles were fed slightly over-satiation a combination of fresh blue mussel and commercial Kuruma prawn pellet daily. Eleven apparently healthy 11 mpe (138.86 ± 14.99 g; n = 8 males, 3 females) in the intermoult phase, exhibiting active behaviour and no missing appendages were starved for 24 h prior to sampling to avoid capturing more transient and feed-associated gut bacteria.

A single lobster (154.6 g; female) 18 days postmoult that exhibited morphological abnormalities was sampled from a mesh cage (approx. 110 L) in a recirculating system (temperature 27.3 ± 0.2 °C; DO 101.4 ± 0.4%; pH 8.10 ± 0.01 and salinity 33.6 ± 0.07 ppt). The animal was fed a combination of blue mussel and Kuruma pellet as described above. The lobster had morphological abnormalities including partial gill exposure, missing appendages (i.e. antennae and 1^{st} – 4^{th} pereiopods) and demonstrated lethargy and minimal response during handling. The individual was considered compromised and used for comparison with the apparently healthy lobsters.

2.3.2 Sample collection

Aseptic techniques were used during lobster dissections and sampling. J1 (8 dpe) juveniles were euthanised in seawater ice slurry for approximately 1 min and surface sterilised in 70% ethanol for 15 s. An inverted Y-shaped incision was made on the dorsal surface of the carapace and the hepatopancreas was excised. The hindgut was extracted from the anterior region of the abdomen after tail fan excision and cephalothorax detachment.

The 11 mpe and abnormal lobsters were euthanised in seawater ice slurry for a minimum of 5 min. The arthrodial membrane at the base of the fifth pereiopod was surface sterilised with 70 % ethanol and haemolymph was extracted using an ice-chilled syringe (27 G needle) containing modified citrate-EDTA buffered anticoagulant (400 mM NaCl, 0.1 M glucose, 30 mM trisodium citrate, 26 mM citric acid, 20 mM EDTA, pH 4.6) (Rodríguez-Ramos et al., 2011). An inverted Y-shaped cut was made on the dorsum of the carapace to extract the midgut and hepatopancreas. The dorsum of the abdomen was incised to extract the hindgut. All the digestive tract samples were put in sterile 0.85 % physiological saline until further processing.

2.3.3 Bacterial culture

Diluted haemolymph samples were spread plated onto Zobell marine agar (ZMA; Amyl Media Pty. Ltd., Dandenong, Australia) and incubated at 28 °C for 136 h. The hepatopancreas, midgut and hindgut were homogenised in 0.85 % physiological saline using micropestles and spread plated onto ZMA and incubated at 28 °C for up to 72 h. Colonies with different morphologies were selected and cultured to purity on ZMA, and re-cultured in Shieh broth (Amyl Media; 5 g L^{-1} bacteriological peptone, 0.1 g L^{-1} sodium pyruvate, 0.01 g L^{-1} sodium acetate, 0.5 g L^{-1} yeast extract, 0.01 g L^{-1} citric acid in 900 mL of 0.2 µm membrane-filtered seawater and 100 mL distilled water) at room temperature and 28 °C for 48 - 120 h on a shaker until turbid. For each pure culture, 1 mL of the turbid broth was cryopreserved in 25 % (v/v) glycerol (-80 °C) for future studies and another 1 mL was used for bacterial identification.

2.3.4 Total nucleic acid (TNA) extraction

For each turbid broth, 1 mL was centrifuged at 16,000 *g* for 5 min. The supernatant was discarded before adding 400 µL of urea extraction buffer (4 M urea, 1 % sodium dodecyl sulfate, 0.2 M sodium chloride, 1 mM sodium citrate; pH 8.2) and 1 µL of proteinase K (Bioline Pty. Ltd., NSW, Australia), heated at 60 °C for 15 min (with 20 s of vortexing every 5 min) and incubated on ice for 5 min. An aliquot of 250 µL 7.5 M ammonium acetate (Sigma-Aldrich Co., MO, USA) was added and vortexed for 20 s. Samples were centrifuged at 12,000 *g* for 5 min at 18 °C. 750 µL of pink co-precipitant (Bioline Pty. Ltd., NSW, Australia) with 0.02 µg μL^{-1} isopropanol was added to the supernatant and inverted 40 times. The mixture was centrifuged at 16,000 *g* for 15 min. The pellet was rinsed with 500 µL of 70 % ethanol twice before being resuspended in 50 µL of molecular grade water.

2.3.5 Sequencing of 16S rRNA gene

PCR reactions (20 µL) contained 10 µL of 2 × MyTaq HS mix, 400 nM of each 27F (5’ - AGA GTT TGA TCM TGG CTC AG - 3’) and 1492R (5’ - GGT TAC CTT GTT ACG ACT T - 3’) 16S rRNA gene primers and 1 µL of TNA extract. The PCR was conducted in a C1000^{TM} Thermal Cycler (Bio-Rad Laboratories Inc., USA) with the thermal cycling program of: initial melting for 3 min at 95 °C; 30 cycles of denaturation for 10 s at 95 °C; annealing for 30 s at 55 °C; extension for 30 s at 72 °C; and a final extension for 3 min at 72 °C. PCR products were examined on a 1 % agarose gel.

PCR products were purified using SureClean (Bioline Pty. Ltd., NSW, Australia) according to the manufacturer’s instructions before measuring the concentrations using a Qubit fluorometer (Invitrogen, Life Technologies Australia Pty. Ltd., VIC, Australia). The purified product with 27F primer was prepared for each sample and sent to Macrogen Inc. (Seoul, Korea) for Sanger sequencing. The resulting sequences were trimmed to 1 % chance of an error per base using Geneious 8.1.7 software employing the 16S Biodiversity Tool for taxonomic classification and comparison (with confidence estimate) to sequences in the Ribosomal Database Project (RDP).

2.4 Results

2.4.1 Overview of microbial diversity

The culture-based microbial diversity in the circulatory and digestive systems of *P. ornatus* is provided in Tables 2-1 and 2-2. Sequence reads ranged from 135 to 1162 bp (mean 1040 bp) with most greater than 1000 bp. Sequences were assigned to known genera with 34 – 100 % confidence, at a mean of 86 %.

At the class level, bacteria recovered from the circulatory and digestive systems included Alphaproteobacteria (*Rhodobacteraceae*, *Cohaesibacteraceae*), Gammaproteobacteria (*Vibrionaceae*, *Alteromonadaceae*, *Pseudomonadaceae*, *Pseudoalteromonadaceae*, *Halomonadaceae*), Flavobacteriia (*Flavobacteriaceae*), Bacilli (*Staphylococcaceae*, *Bacillaceae*) and Actinobacteria (*Microbacteriaceae*). Three classes of bacteria (Gammaproteobacteria, Alphaproteobacteria and Bacilli) were associated with lobster hepatopancreas, midgut and hindgut, while the haemolymph comprised a more diverse range of bacteria belonging to Gammaproteobacteria, Alphaproteobacteria, Bacilli, Flavobacteriia and Actinobacteria.

2.4.2 Bacteria in the circulatory system

Bacteria isolated from the haemolymph of 11 mpe lobsters belonged to ten genera, dominated by members of the *Rhodobacteraceae* (eg. *Ruegeria*, *Silicibacter*, *Roseovarius*) and *Flavobacteriaceae* (Table 2-1). Bacteria belonging to the genera *Marinobacter*, *Halomonas*, *Staphylococcus* and *Microbacterium* were recovered from healthy lobsters only. In contrast, the individual abnormal lobster appeared to harbour a higher diversity of cultured bacteria when compared to healthy individuals, including isolates belonging to *Rhodobacteraceae* (4 genera), *Flavobacteriaceae* (2 genera), *Cohaesibacteraceae* (1 genus) *and Vibrionaceae* (1 genus).

2.4.3 Bacteria in the digestive system

Three bacterial genera were detected in the hepatopancreas (*Vibrio*, *Pseudoalteromonas*, *Bacillus*) and two genera each in the midgut (*Vibrio*, *Pseudomonas*) and hindgut (*Vibrio*, *Shimia*) (Table 2-2). The majority of isolates recovered were affiliated with members of *Vibrionaceae*. *Bacillus* and *Shimia* isolates were associated only with 8 dpe juveniles, while *Pseudomonas* isolates were recovered from 11 mpe lobsters only. *Vibrio*-affiliated isolates were dominant in the hepatopancreas, midgut and hindgut of the abnormal lobster.

Table 2-1. Haemolymph-associated bacteria from 11 mpe (n = 9) and abnormal (n = 1) lobsters.

Bacteria (family)	Bacteria (genus)	Lobster ID	No. of isolates	Mean confidence, %[a] (Sequence length, bp)
Rhodobacteraceae	*Ruegeria*	11mpe-1	1	100 (1054)
		11mpe-2	1	99 (1055)
		11mpe-3	2	98 (1110), 100 (1111)
		11mpe-4	2	100 (1066), 99 (1057)
Rhodobacteraceae	*Ruegeria*	11mpe-2	1	84 (1112)
Rhodobacteraceae	*Ruegeria*	11mpe-4	1	98 (1052)
		11mpe-5	1	97 (1061)
Rhodobacteraceae	*Silicibacter*	11mpe-2	1	76 (1055)
		11mpe-3	2	54 (1150), 61 (1098)
		11mpe-4	2	40 (1158), 56 (1161)
		11mpe-6	1	57 (1056)
Rhodobacteraceae	*Silicibacter*	11mpe-3	2	45 (1055), 53 (1061)
Rhodobacteraceae	*Roseovarius*	11mpe-4	1	98 (1159)
Rhodobacteraceae	*Roseovarius*	11mpe-6	1	98 (1116)
Rhodobacteraceae	*Roseovarius*	Abnormal-1	5	56 (1071), 62 (1058), 62 (1058), 75 (1059), 95 (1034)
Rhodobacteraceae	*Thalassobius*	Abnormal-1	1	84 (1050)
Rhodobacteraceae	*Shimia*	Abnormal-1	1	100 (1107)
Rhodobacteraceae	*Labrenzia*	Abnormal-1	1	100 (1061)
Flavobacteriaceae	*Muricauda*	11mpe-3	1	88 (981)
Flavobacteriaceae	*Maribacter*	11mpe-4	1	100 (1071)
Flavobacteriaceae	*Arenibacter*	11mpe-7	1	37 (1162)
Flavobacteriaceae	*Kordia*	Abnormal-1	1	39 (1075)
Flavobacteriaceae	*Meridianmaribacter*	Abnormal-1	1	34 (1067)
Microbacteriaceae	*Microbacterium*	11mpe-7	1	90 (135)
Alteromonadaceae	*Marinobacter*	11mpe-8	1	100 (973)
Halomonadaceae	*Halomonas*	11mpe-9	1	100 (1095)
Staphylococcaceae	*Staphylococcus*	11mpe-9	1	100 (1097)
Vibrionaceae	*Vibrio*	Abnormal-1	1	100 (1103)
Cohaesibacteraceae	*Cohaesibacter*	Abnormal-1	1	99 (491)

[a] Sequences were identified to the closest relative in Ribosomal Database Project.

Table 2-2. Digestive tract-associated bacteria from 11 mpe (n = 5), 8 dpe (n = 2) and abnormal (n = 1) lobsters.

Sample	Bacteria (family)	Bacteria (genus)	Lobster ID	No. of isolates	Mean confidence, %[a] (Sequence length, bp)
Hepatopancreas	*Vibrionaceae*	*Vibrio*	8dpe-1	1	100 (958)
	Vibrionaceae	*Vibrio*	11mpe-8	1	100 (845)
	Vibrionaceae	*Vibrio*	Abnormal-1	1	100 (1002)
	Vibrionaceae	*Vibrio*	Abnormal-1	1	74 (1017)
	Pseudoalteromonadaceae	*Pseudoalteromonas*	11mpe-10	1	100 (1089)
	Pseudoalteromonadaceae	*Pseudoalteromonas*	11mpe-11	1	100 (1095)
	Bacillaceae	*Bacillus*	8dpe-2	1	100 (1093)
Midgut	*Vibrionaceae*	*Vibrio*	11mpe-4	1	93 (1128)
			Abnormal-1	2	93 (1057), 100 (999)
	Pseudomonadaceae	*Pseudomonas*	11mpe-8	1	94 (1091)
Hindgut	*Vibrionaceae*	*Vibrio*	8dpe-1	1	100 (958)
	Vibrionaceae	*Vibrio*	8dpe-2	1	100 (1041)
	Vibrionaceae	*Vibrio*	11mpe-6	1	100 (1109)
	Vibrionaceae	*Vibrio*	Abnormal-1	1	100 (1068)
	Rhodobacteraceae	*Shimia*	8dpe-1	1	100 (1112)

[a] Sequences were identified to the closest relative in Ribosomal Database Project.

2.5 Discussion

The presence of culturable bacteria in the haemolymph of apparently healthy 11 mpe lobsters is consistent with previous studies that indicate bacteria are natural components of the circulatory system and does not necessarily infer disease (Evans et al., 2003; Wang and Wang, 2015). In the present study, ten bacterial genera belonging to five classes were cultured from the haemolymph of apparently healthy lobsters indicative of significant bacterial diversity.

Some of the bacterial genera associated with the lobster haemolymph have also been detected in other crustaceans (Scott and Thune, 1986; Wong et al., 1995; Bartlett et al., 2008; Battison et al., 2008). The family *Rhodobacteraceae* and associated genera *Ruegeria*, *Silicibacter* and *Roseovarius* were recovered most frequently in this study, and these groups have been often reported from the seawater environment and marine invertebrates including oysters and clams (Pujalte et al., 2014). Interestingly, *Ruegeria* spp. have also been isolated from cultured *P. ornatus* phyllosomas (Goulden, 2012). The abundance of *Ruegeria* spp. could be a consequence of their marine cosmopolitan distribution (Gram et al., 2010), propensity to form symbiotic relationships with aquatic animals (Santisi et al., 2015) or functional involvement in carbon and sulphur cycles in the marine environment (Wagner-Döbler and Biebl, 2006; Pujalte et al., 2014). Another important bacterial family associated with lobster haemolymph was *Flavobacteriaceae* which included genera *Arenibacter*, *Muricauda* and *Maribacter*, and these are also associated with marine ecosystems (Bernardet and Nakagawa, 2006; Bowman, 2006; Meziti et al., 2012). *Flavobacteriaceae*-affiliated bacteria are reputed to produce enzymes that degrade a range of organic compounds and forming symbiotic relationships with insects (Bernardet and Nakagawa, 2006). Another genus isolated in this study was *Staphylococcus*, which was also retrieved from the haemolymph of wild American lobster *H*. *americanus* (Bartlett et al., 2008), pond-reared red claw crayfish *Cherax quadricarinatus* (Wong et al., 1995) and red swamp crawfish *Procambarus clarkii* (Scott and Thune, 1986). Although *Halomonas* and *Microbacterium* were detected in the haemolymph of apparently healthy lobsters in this study, these genera have previously been found in the gut of other healthy crustaceans including *H. americanus* (Battison et al., 2008) and red cherry shrimp *Neocaridina denticulata* (Cheung et al., 2015). The culture library constructed in this study can be used for further screening assays as these isolates may have commercial value not just in lobster health, but perhaps human health (Wietz et al., 2010).

In contrast to healthy lobsters, the abnormal lobster harboured *Vibrio* isolates in the haemolymph. *Vibrio* spp. have been reported in the haemolymph of both healthy and diseased lobsters (Shields, 2011). *Vibrio* spp. were in the haemolymph of apparently healthy lobsters including *P. ornatus* (Evans et al., 2003), scalloped spiny lobster *P. homarus* (Raissy et al., 2011), western rock lobster *P. cygnus* (Evans et al., 1996), southern rock lobster *J. edwardsii* (Handlinger et al., 2006) and *H. americanus* (Bartlett et al., 2008). On the other hand, many vibrios are considered opportunistic bacteria, and may cause disease when animals are stressed or immunocompromised (Immanuel et al., 2006). For example, *V. alginolyticus* was isolated in septicaemic *P. homarus* with shell disease (Abraham et al., 1996). Another bacterial isolate of interest only detected in the haemolymph of the abnormal lobster was *Thalassobius.* Strains of this genus have been detected in the haemolymph of *H. americanus* with epizootic shell disease (ESD) lesions (Quinn et al., 2013), and are considered one of the primary pathogens for ESD (Quinn et al., 2012). While the abnormal lobster did not have deep cuticular erosions like lobsters with ESD, the animal exhibited exposed gills and missing limbs, which could be portals for bacterial entry. The only bacterial genus found in the haemolymph of both apparently healthy lobsters and the abnormal individual was *Roseovarius*. Overall, the culturable bacterial community results associated with the abnormal individual could indicate a dysbiotic shift whereby a higher abundance of potential opportunistic pathogens were detected. However, a larger community fingerprint (i.e bacterial metagenomic analysis) is required to validate this hypothesis.

Vibrio-affiliated isolates were most frequently recovered from the digestive tract (hepatopancreas, midgut, hindgut) of both 8 dpe and 11 mpe apparently healthy lobsters. This could suggest vibrios constitute a major part of the natural microbiota of the lobster digestive system. Some of the perceived functions of vibrios include producing enzymes to digest chitin and producing antibiotics to inhibit other bacteria from colonising the gut (Thompson et al., 2004). In addition to *Vibrio*, isolates belonging to the genera *Pseudomonas*, *Pseudoalteromonas* and *Bacillus* were also retrieved from the digestive tract of these animals, consistent with other studies on apparently healthy lobsters. For example, *Vibrio* sp. and *Pseudoalteromonas* sp. were identified in the gut of reared *N. norvegicus* (Meziti et al., 2012) and *H. americanus* (Battison et al., 2008). Likewise in wild lobsters, *Vibrio*, *Pseudomonas* and *Bacillus* were affiliated with the gut of *P. homarus* (Immanuel et al., 2006) and Japanese spiny lobster *P. japonicus* (Sugita et al., 1987). Therefore, it is likely these bacterial genera could harbour beneficial strains implicated in health. In contrast, *Vibrio* spp. have been isolated from wild *J. edwardsii* (Evans, 1999) and reared *H.*

americanus (Battison et al., 2008) in association with gut pathologies. In the present study, there was no relationship between gross digestive pathology and the presence of vibrios in the abnormal lobster. However, there was a possibility that vibrios could have spread from the digestive tract into the circulatory system given the physiological stress imposed by the morphological shortcomings of this animal. The missing appendages might have compromised feeding behaviours leading to nutritional deficiencies and reduced immune response.

The microbial communities in the digestive tract were distinct from those in the haemolymph of the apparently healthy lobsters in this study. Host organisms can have a 'habitat filter' whereby tissue, organ or system-specific resources, conditions and immune responses only permit the residence and growth of certain bacteria (Costello et al., 2012). Some filtering mechanisms of the lobster digestive system include the gastric sieve, digestive enzymes and mucosal barriers (Hopkin and Nott, 1980; Battison et al., 2008; Raissy et al., 2011). In the haemolymph, crustaceans control the growth of microbiota using antimicrobial peptides and lectins (Wang and Wang, 2015). Moreover, bacterial populations (including pathogens) can be prevented from colonising certain tissues by other bacteria competing for resources and space (Costello et al., 2012).

There was some degree of variance in the confidence levels (34 – 100 %) of sequence assignment to known genera in the present study. Prior to the availability of 16S rRNA gene sequencing or genotyping, microbes were classified according to phenotypic characteristics (Clarridge, 2004). With the advent of bioinformatics and sequence database repositories such as RDP, a bacterium can now be identified to the closest relative whose 16S rRNA sequence is present in database. As the genotypic and phenotypic databases of microbes expands, this is likely to result in numerous reclassifications and changes in nomenclature. As a consequence, this can make comparisons of bacterial species in modern studies to those identified in earlier studies problematic. Moreover, there can be significant intraspecies variation of the 16S rRNA gene meaning databases need to greatly expand to ensure correct identification of each species (Clarridge, 2004) or studies would need to include multilocus sequencing of additional housekeeping genes for greater resolution to the species level (Cano-Gomez et al., 2011). As such, there is greater confidence assigning bacteria to the genus or family level as opposed to species.

This study shows that the haemolymph of apparently healthy *P. ornatus* is not sterile which is consistent with other healthy crustaceans (Shields et al., 2015; Wang and Wang, 2015). These preliminary findings provide a strong basis for more in-depth studies using molecular techniques of bacterial community analyses that are more sensitive and enable characterisation of non-culturable bacteria. Future studies employing next generation sequencing and quantitative PCR will allow comprehensive analyses of microbial diversity and load in the haemolymph and gut.

Chapter 3 Haemolymph microbiome of the cultured spiny lobster *Panulirus ornatus* at different temperatures

Part of the research contained within this chapter has been published as Ooi, M.C., Goulden, E.F., Smith, G.G., Bridle, A.R., 2019. Haemolymph microbiome of the cultured spiny lobster *Panulirus ornatus* at different temperatures. Sci. Rep. 9, 1677.

3.1 Abstract

Lobsters have an open circulatory system with haemolymph that may contain microorganisms even in the healthy individuals. Understanding the role of these microorganisms becomes increasingly important particularly for the diagnosis of disease as the closed life cycle aquaculture of the spiny lobster *Panulirus ornatus* nears commercial reality. This study aimed to characterise haemolymph responses of healthy cultured *P. ornatus* juveniles at control (28 °C) and elevated (34 °C) temperatures. This was assessed by measuring immune parameters (total granulocyte counts, total haemocyte counts, clotting times), and culture-independent (pyrosequencing of haemolymph DNA) and culture-dependent (isolation using nonselective growth medium) techniques to analyse bacterial communities from lobster haemolymph sampled on days 0, 4 and 6 post-exposure to the temperature regimes. Elevated temperature (34 °C) affected lobster survival and total granulocyte counts as well as diversity, load and functional potential of the haemolymph bacterial community. Pyrosequencing analyses showed that the core haemolymph microbiome consisted of phyla Proteobacteria and Bacteriodetes. Overall, culture-

independent methods captured a higher bacterial diversity and load when compared to culture-dependent methods, however members of the *Rhodobacteraceae* were strongly represented in both analyses. This is the first comprehensive study providing comparisons of haemolymph bacterial communities from healthy and thermally stressed cultured juvenile *P. ornatus* and has the potential to be used in health monitoring programs.

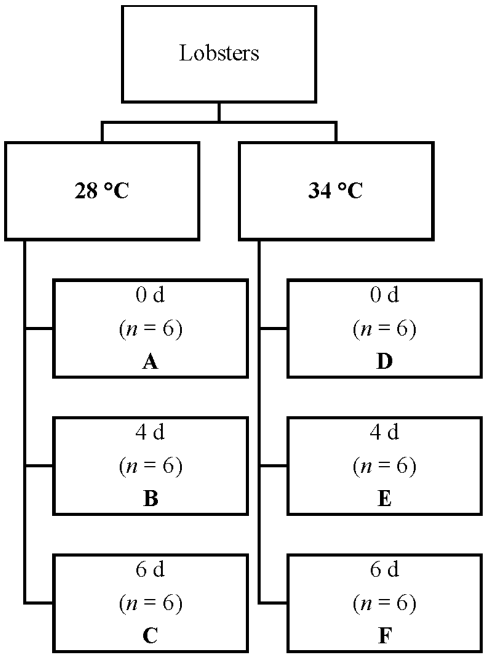
3.2 Introduction

The ornate spiny lobster *Panulirus ornatus* is sought after as a high-end seafood product (Jeffs, 2010). It has been produced by sea-cage aquaculture for more than 25 years in parts of south east Asia, where seedstock is sourced from the wild and grown to market size over an 18 month period (Jeffs, 2010). However, since the late 1990s the University of Tasmania has been developing closed life cycle production technologies to breed and grow *P. ornatus* from egg to adult. This research has now spawned a company to commercialise the technology (UTAS Nexus Aquasciences - UNA), and is expected to significantly change the landscape of lobster industries by reducing wild seedstock harvesting and bolstering of seafood production and stock enhancement programs. The ongoing success of industrialised closed life cycle production systems for lobsters will rely on better knowledge of cultured lobster health, with particular focus on non-lethal haemolymph sampling and microbiomic information that allows for health status assessment.

Environmental stressors, in particular temperature, can significantly impact animal health and productivity. For poikilothermic lobsters, ambient temperatures directly affect oxygen utilisation, metabolism, growth and moulting (Crear and Forteath, 2000; Moullac and Haffner, 2000), whereas elevated temperatures can drastically impact the lobster immune system (Shields et al., 2006). Sea-cage grown *P. ornatus* is preferentially cultured in shallow coastal bays, where the animals are protected from strong wind or wave action, but the minimal water flushing in these areas subject animals to sporadic water temperatures well above optimal (25 – 31 °C) (Jones, 2010). We explored the upper thermal limit of *P. ornatus* in a pilot study using a flow-through water set-up and found mortalities began at 35 °C after 3 days of exposure (unpub. data). This information becomes significant in the context of climate change, as elevated sea temperatures are becoming more frequent and have the potential to cause higher lobster mortality rates (Norman-López et al., 2013).

Temperature stress may also significantly affect host-microbiota interactions in lobsters and other decapods, including those within the circulatory system (Tubiash et al., 1975; Scott and Thune, 1986). Most invertebrates have open circulatory systems in which haemolymph (equivalent to blood) is actively circulated around the haemocoelic cavity (Martin and Hose, 1995) and returns to the heart via the ostia (Wirkner et al., 2013). Healthy invertebrates can harbour a range of bacteria in their blood without exhibiting systemic disease and associated clinical signs (Desriac et al., 2014; Wang and Wang, 2015). This condition is termed asymptomatic bacteraemia (Evans et al., 2014) and has been found so far in crustaceans (Wang and Wang, 2015) and molluscs (Desriac et al., 2014; Lokmer and Wegner, 2015). Bacteria in the haemolymph of crustaceans may positively impact host health by modulating the immune response, producing antimicrobial substances and competing with pathogens (Wang and Wang, 2015). However, temperature stress may cause dysbiosis of haemolymph communities leading to proliferation and increased bacterial load (septicaemia), which has been reported in red swamp crawfish *Procambarus clarkii* (Scott and Thune, 1986) and blue crab *Callinectes sapidus* (Tubiash et al., 1975).

Previously, bacteria were found in the haemolymph of 5 – 100% of lobster species sampled by culture-dependent methods, including *P. ornatus* (Evans et al., 2003), western rock lobster *P. cygnus* (Evans et al., 1996), scalloped spiny lobster *P. homarus* (Mary Leema et al., 2010), southern rock lobster *Jasus edwardsii* (Handlinger et al., 2006) and American lobster *Homarus americanus* (Bartlett et al., 2008). However, recently there has been a shift to employ culture-independent techniques (e.g., targeted and non-targeted sequencing) that have increased sensitivity and that have been used to discover bacteria in the haemolymph in 100% of lobsters(Quinn et al., 2013). Although bacteria have been detected in the haemolymph of lobsters, limited information exists for the bacterial composition and diversity associated with healthy lobsters (Quinn et al., 2013), which makes the identification of potentially invasive and pathogenic microorganisms difficult. Furthermore, the effect of temperature on the bacterial communities and lobster immune responses is unknown. The present study aimed to characterise and quantify (1) haemolymph immune responses (e.g., total haemocyte and granulocyte counts, clotting times) and (2) haemolymph microbial communities (e.g., diversity, composition, core microbiome) using a combination of culture-dependent and culture-independent techniques (next generation sequencing, quantitative PCR) of juvenile *P. ornatus* exposed to optimal culture temperature (28 °C) and temperature stress (34 °C) just below the perceived thermal lethal limit (Figure 3-1). Animals were exposed to 6 days of the thermal regimes to ensure a stress response but short enough to avoid mortality.



Sampling parameters	Sample group
Immunity (haemocyte counts, granulocyte counts, clotting time)	A - F
Bacterial diversity	A, C, F
Bacterial load	A - F

Figure 3-1. Experimental design showing sample groups and sampling parameters.

3.3 Materials and methods

3.3.1 Experimental design

Panulirus ornatus were reared from embryos that hatched as larvae and metamorphosed into juveniles at the University of Tasmania's Institute for Marine and Antarctic Studies (IMAS) located in Hobart, Australia. Culture methods were based on modified protocols from Jensen et al. (2013) and Fitzgibbon and Battaglene (2012). Juveniles were cultured communally in 4,000 L fiberglass tanks within a recirculating system. Water quality parameters during culture were temperature 28.1 ± 0.5 °C, pH 8.05 ± 0.1, salinity 34 ± 0.5 ppt and dissolved oxygen 95 ± 5%. Juveniles were fed once daily fresh blue mussel (*Mytilus galloprovincialis*) or squid (*Nototodarus gouldi*) at a rate of approximately 10% wet weight of total tank biomass. For this experiment, 44 lobsters (162.7 ± 4.6 g) were placed individually in oyster mesh cages and distributed in 6 × 600 L fiberglass tanks (7 - 8 lobsters per tank) where they were acclimated for 24 h in flow-through sea water (temperature 28.3 ± 0.03 °C; dissolved oxygen 92.8 ± 1.1%). The experiment started after this acclimation period. In three of the tanks, lobsters were exposed to thermal stress by increasing the temperature from 28 to 34 °C (33.9 ± 0.02 °C) over 28 h (Figure 3-1). The remaining three tanks (control juveniles) were maintained at 28°C (28.0 ± 0.02 °C). Animals were not fed during the six-day experiment.

3.3.1.1 Haemolymph sampling

On 0, 4 and 6 days post-treatment (dpt), six randomly chosen lobsters from each thermal treatment were euthanised in a seawater ice slurry for 5 min before haemolymph was sampled for immune and bacterial assays. Haemolymph was aspirated from the base of pereiopods and heart (Evans, 2003) using a sterile ice-chilled syringe and needle. For all assays except clotting time and spread plate culture, the syringe was pre-filled with an equal volume of anticoagulant (modified from (Rodríguez-Ramos et al., 2011); 400 mM NaCl, 0.1 M glucose, 30 mM trisodium citrate, 26 mM citric acid, 40 mM EDTA). Lobsters that died during the experiment were not sampled as haemolymph clots quickly after death rendering separation of plasma from haemocytes unfeasible for downstream analyses, but dead lobsters were counted for survival analysis (n = 22 for each thermal treatment).

3.3.2 Immune parameters

Haemocytes including hyalinocytes (phagocytosis) and granulocytes (encapsulation, prophenoloxidase system, cytotoxicity) were measured as they play major roles in the crustacean immune response (Johansson et al., 2000). Total haemocyte counts and May-Grunwald and Giemsa staining of haemolymph smears were conducted according to Evans (2003). Large granular and small granular haemocytes were counted together as granulocytes (Sritunyalucksana et al., 2005). The percentage of granulocytes was found by expressing the fraction of total granulocytes from 100 counted total haemocytes. Clotting times were analysed according to Evans (2003) and samples that did not clot before 60 s were recorded as no clot and excluded from statistical analysis.

3.3.3 Culture-dependent bacterial analyses

Aliquots of 100 µL of haemolymph from each lobster was plated onto Zobell marine agar (Amyl Media Pty. Ltd., Dandenong, Australia). Plates were incubated at 28 °C for up to 7 d (to allow slower growing bacteria to colonise when plates were not too crowded by overgrowth) and the number of colony forming units (CFUs) were counted. All colonies from each lobster reared at 28 °C or 34 °C on 6 dpt were isolated and cultured in Shieh broth (Amyl Media; 5 g L^{-1} bacteriological peptone, 0.1 g L^{-1} sodium pyruvate, 0.01 g L^{-1} sodium acetate, 0.5 g L^{-1} yeast extract, 0.01 g L^{-1} citric acid in 900 mL of sea water and 100 mL distilled water), pooled according to treatment group and stored in urea extraction buffer (4 M urea, 1% sodium dodecyl sulfate, 0.2 M sodium chloride, 1 mM sodium citrate; pH 8.2) prior to PCR and sequencing (Section 4.4.3).

3.3.4 Culture-independent bacterial analyses

3.3.4.1 Sampling

One millilitre of anticoagulated haemolymph from each lobster was centrifuged at 100 *g* for 5 min to separate plasma from haemocytes. The supernatant was centrifuged at 10,000 *g* for 10 min to collect bacteria from the plasma. All but 100 µL of the supernatant was removed before adding 400 µL of urea extraction buffer and sample storage at -20 °C until further processing.

3.3.4.2 Total nucleic acids (TNA) extraction

Haemolymph samples were vortexed in urea extraction buffer and 100 µg of proteinase K (Bioline Pty. Ltd., NSW, Australia), heated at 55 °C for 1 h (vortexed every 5 min) and incubated on ice for 5 min. Ammonium acetate (7.5M; Sigma-Aldrich Co., MO, USA) was added to a final concentration of 2.5 M, vortexed for 30 s and centrifuged at 14,000 *g* for 5 min (18 °C). The supernatant was mixed by inversion with 1 mL of isopropanol with 0.02 µg μL^{-1} pink co-precipitant (Bioline) and centrifuged at 16,000 *g* for 30 min. The pellet was rinsed with 500 µL of 70% ethanol twice and resuspended in 50 µL of buffered water (0.05% Triton X-100, 10 mM TRIS pH 7). Protocol modifications were made to extract TNA from pooled bacterial colonies. Each pool of colonies was put on ice for 5 min. Ammonium acetate (7.5 M) was added to a final concentration of 2.5 M, vortexed for 30 s and centrifuged at 12,000 *g* for 5 min (18 °C). The supernatant was mixed with an equal volume of isopropanol with pink co-precipitant and centrifuged at 12,000 *g* for 15 min. The pellet was rinsed with 70% ethanol twice before resuspension in 100 µL of buffered water. For purification, each TNA extract was added with an equal volume of 19% polyethylene glycol, mixed and incubated at room temperature for 15 min then centrifuged at 14,000 *g* for 15 min (18 °C). The pellet was rinsed with 200 µL of 70% ethanol, centrifuged for 14,000 *g* for 3 min and resuspended in 50 µL of buffered water.

3.3.4.3 PCR and pyrosequencing

A nested PCR approach was used due to the occurrence of low molecular weight non-specific PCR products when TNA extract from haemolymph was used directly in PCR with pyrosequencing primers. The primary PCR mixture consisted of 10 µL of 2 × MyTaq HS mix (Bioline), 200 nM each of 27F (5' - AGAGTTTGATCMTGGCTCAG - 3') and 1492R (5' - GGTTACCTTGTTACGACTT - 3') 16S rRNA gene primers and 2 µL of purified TNA. The PCR was conducted using a C1000™ Thermal Cycler (Bio-Rad Laboratories Inc., USA) with the following thermal cycling program: initial melting for 3 min at 95 °C; 20 cycles of denaturation for 10 s at 95 °C, annealing for 30 s at 55 °C, extension for 30 s at 72 °C; and a final extension for 3 min at 72 °C. Barcoded primers (Ooi et al., 2017) were used in the secondary PCR to amplify V1 to V3 hypervariable regions of the 16S rRNA gene. The secondary PCR contained 10 µL of 2 × MyTaq HS mix, 250 nM each of forward and reverse 16S rRNA gene primers and 2 µL of 1:10 diluted primary PCR products. The thermal cycling program for secondary PCR was initial denaturation for 3 min at 95 °C; 25 cycles of denaturation for 10 s at 95 °C, annealing for 30 s at 58 °C, extension for 10 s at 72 °C; and a final extension for 2 min at 72 °C. PCR products were examined using 1.5% agarose gel electrophoresis. All PCRs included negative extraction and amplification controls (no band on gel).

As there was no non-specific product in PCR of pooled bacterial colonies, direct PCR was used. The PCR contained 10 µL of 2 × MyTaq HS mix, 300 nM each of barcoded forward and reverse 16S rRNA gene primers and 2 µL of 1:10 diluted TNA extract. The thermal cycling program consisted of initial denaturation for 3 min at 95 °C; 30 cycles of denaturation for 10 s at 95 °C, annealing for 30 s at 58 °C, extension for 15 s at 72 °C; and a final extension for 2 min at 72 °C. PCR products were examined using 1.5% agarose gel electrophoresis.

All PCR products were concentrated using isopropanol precipitation before purification. Ammonium acetate was added to PCR products to reach a final concentration of 2.5 M. An equal volume of isopropanol with pink co-precipitant was put into the mixture, incubated on ice for 30 min and centrifuged for 16,000 *g* for 30 min. The pellet was washed twice with 70% ethanol and resuspended in buffered water. Concentrations of PCR products were determined using a Qubit™ fluorometer (Invitrogen™, Life Technologies Australia Pty. Ltd., VIC, Australia). Twenty five nanograms of barcoded PCR amplicon per sample (18 culture-independent and 2 culture-dependent samples were selected due to cost limitation) was pooled. The pool was purified using SureClean (Bioline) according to the manufacturer's instructions and quantified using Qubit. A 100 µL suspension containing 2 ng μL^{-1} of PCR amplicons was sent to Macrogen Inc. (Seoul, Korea) for pyrosequencing (454 GS-FLX Titanium, Roche, USA).

3.3.4.4 Quantitative PCR (qPCR)

The TNA of *Yersinia ruckeri* (serotype 01b, strain UTYR001) was extracted and diluted in a ten-fold series to be used as standards. The qPCR reaction (10 uL) consisting of 5 µL of 2 × MyTaq HS mix, 400 nM each of YrF (5' – AACCCAGATGGGATTAGCTAGTAA - 3') and YrR (5' – GTTCAGTGCTATTAACACTTAACCC – 3') 16S rRNA gene-based primers (Carson and Wilson, 2009), 100 nM of hydrolysis probe (5' HEX – AGCCACACTGGAACTGAGACACGGTCC – 3' BHQ1) (Ghosh et al., 2016) and 2 µL of *Y. ruckeri* TNA was processed in CFX Connect® Real-Time System (Bio-Rad Laboratories Inc., USA). The qPCR program was 95 °C for 3 min and 40 cycles of 95 °C for 5 s and 60 °C for 30 s.

As the copy number of 16S rRNA gene varies between bacteria, single copy RNA polymerase beta subunit (*rpoB*) gene was used for estimating bacterial load. Two microlitres of standards (*Y. ruckeri*) and haemolymph TNA (1:2 dilution) were included in qPCR reactions (10 µL) consisting of 5 µL of 2 × MyTaq HS mix with SYBR, 400 nM each of *rpoB*1698f and *rpoB*2041r primers (Dahllöf et al., 2000). Duplicates of templates, standards, negative extraction and amplification controls were run in a 96 well plate. The reaction conditions were 95 °C for 3 min and 40 cycles of 95 °C for 10 s, 55 °C for 20 s and 72 °C for 10 s. Melt curve analysis was performed at 95 °C for 10 s and 58 °C for 5 s to allow reannealing before melting from 72 to 92 °C (0.5 °C increment per 5 s). The amplification efficiency of the standard curve was 91.0 %.

3.3.5 Data analyses

For total haemocyte and granulocyte counts, and clotting time a normality test was performed before conducting either parametric (independent samples *t* test) or in the case of non-normal data, non-parametric (Mann-Whitney U test) statistic was used to compare lobsters reared at 28 °C and 34 °C at each time point. Similarly for alpha diversity indices and bacterial load, a normality test was conducted before parametric or non-parametric statistics. Data were analysed using SPSS v20. A *P* value of less than 0.05 was considered significant for all the statistical analyses.

For bacterial diversity, Geneious 8.1.7 (Kearse et al., 2012) was used to demultiplex the sequence file to individual samples based on the barcodes and to trim primers. The sequences were exported to the Data Intensive Academic Grid computational cloud (DIAG, 2016) for use with the CloVR pipeline for 16S rRNA amplicon analysis. Chimeric and poor quality (≤ 100 bp, ≥ 2000 bp, homopolymer ≥ 8 bp, and ambiguous bases > 0) sequences were removed by UCHIME and Qiime (White et al., 2011). MOTHUR was used to cluster unique sequences, analyse richness and diversity indices and compute rarefaction curves (White et al., 2011). Operational taxonomic units (OTUs) or clusters of filtered sequences with 97% nucleotide sequence identity were assigned to known bacterial taxa based in the Greengenes database v2013 (McDonald et al., 2012) with a confidence threshold of 0.8 using RDP Bayesian Classifier. Good's coverage was calculated based on the formula of (1 – [number of singleton reads / total number of reads]) × 100 %. A .biom-formatted file from CloVR was uploaded to MicrobiomeAnalyst (Dhariwal et al., 2017) to examine alpha diversity (observed OTUs, Chao1, ACE, Simpson, Shannon), beta diversity, core microbiome, abundance, and functional potential of OTUs in the samples. Low abundance OTUs (≤ 2 counts) with 10% or lower prevalence in samples were removed to facilitate the analysis. Data were normalised by rarefying to the minimum library size, i.e., 2,325. The beta diversity was analysed by Bray Curtis, weighted and unweighted UniFrac distance based PCoA (OTU level) and PERMANOVA. For culture-dependent samples, actual abundance was reported as the count of each OTU was not directly proportional to the total count. MetagenomeSeq (zero-inflated Gaussian fit) compared OTU abundance (mean ± standard error) between two thermal regimes and a false discovery rate-adjusted *P* value (Q) of < 0.05 was considered significant. Functional potential of OTUs was predicted using PICRUSt (Langille et al., 2013). The functional diversity profile was generated from the sum of abundance of each OTU for each KEGG (Kyoto Encyclopedia of Genes and Genomes) metabolism normalised by category size. The functional association analysis was used to demonstrate the KEGG pathways and *P* value of < 0.05 was considered significant. A Venn diagram showing numbers of shared and unique OTUs among samples was generated using InteractiVenn (Heberle et al., 2015).

For bacterial load using qPCR, target amplicons were distinguished from non-specific amplification of primer dimers by melt curve analysis. The target amplicon was not present in negative extraction and amplification controls. The number of 16S rRNA copies in the *Y. ruckeri* standards were determined using CM3 model (Ghosh et al., 2016) in the qPCR package of RStudio (v3.2.3) software. The number of 16S rRNA copies in the standards were converted to the cell equivalents to allow construction of a standard curve for the samples (*rpoB* gene).

3.3.6 Data availability

The sequences generated during the current study are available in the NCBI Sequence Read Archive under BioProject accession PRJNA445187.

3.4 Results

3.4.1 Survival rate and immune parameters

Lobster survival and immune parameters were measured to assess the impact of temperature on *P. ornatus*. There was no mortality until the fourth day of exposure to 34 °C; the survival rates were 94% on 4 dpt and 70% on 6 dpt (Figure 3-2A). No mortality was observed in the 28 °C treatment. The total granulocyte counts of lobsters exposed to 34 °C on 6 dpt dropped significantly when compared to those on 4 dpt ($U = 5.000$, $P = 0.041$) and control animals on 6 dpt ($U = 5.000$, $P = 0.041$) (Figure 3-2B). Total haemocyte counts ranged from $4.8 \times 10^5 – 1.3 \times 10^7$ cells mL^{-1}, however no significant difference ($P > 0.05$) was found between animals exposed to 34 °C and 28 °C (Figure 3-2C). Clotting times did not significantly differ ($P > 0.05$) between juveniles held at either temperature and ranged between 18 – 57 s (Figure 3-2D).

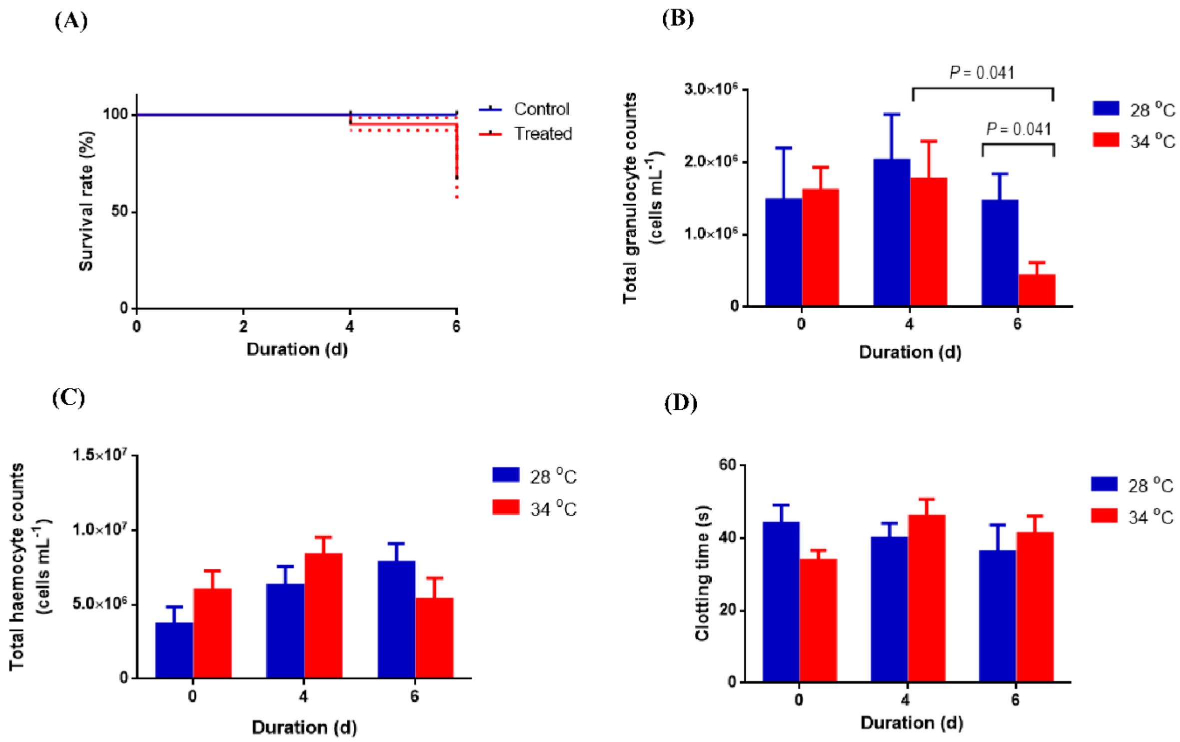


Figure 3-2. (A) Survival rate (%), (B) total granulocyte counts (cells mL^{-1}), (C) total haemocyte counts (cells mL^{-1}) and (D) clotting time (s) of *P. ornatus* juveniles exposed to 28 °C or 34 °C. Each bar represents mean + SEM, n = 6 (except clotting time on days 4 and 6 with n = 4).

3.4.2 Bacterial diversity

3.4.2.1 Summary of pyrosequencing

V1 to V3 hypervariable regions of the bacterial 16S rRNA gene in the haemolymph sample libraries were sequenced. A total of 104,183 reads from 20 samples were obtained after quality filtering and removal of chimeric sequences (Table 3-1). Mean reads per sample was 5,209 and mean read length was 302 bp. Good's coverage ranged from 94.7 to 99.1 % (Table 3-1).

Table 3-1. Sampling depth, richness and alpha diversity indices for haemolymph sequence libraries of juvenile *P. ornatus*.

Sample ID	Lobster no.	Sampling depth									Richness estimators		Diversity indices	
		Raw sequences	Filtered sequences	Obs. OTUs	Good's coverage (%)	Phylum	Class	Order	Family	Genus	Chao1	ACE	Shannon	Simpson
28 °C day 0														
Culture-independent samples														
28C0d1	1	7,532	4,354	109	97.8	6	11	19	18	21	144	145	2.54	0.81
28C0d2	2	4,790	2,660	119	97.2	7	10	16	16	15	130	136	3.71	0.96
28C0d3	3	4,248	2,389	160	96.5	6	12	16	14	18	177	181	4.39	0.98
28C0d4	4	9,229	6,979	230	97.7	8	13	25	30	32	320	280	4.19	0.95
28C0d5	5	9,689	6,978	274	97.1	11	19	32	35	39	344	367	4.33	0.96
28C0d6	6	4,972	3,755	237	94.7	13	19	31	30	34	281	318	4.26	0.97
28 °C day 6														
Culture-independent samples														
28C6d1	1	5,132	3,745	172	96.8	7	11	22	24	21	198	204	3.76	0.93
28C6d2	2	8,588	6,261	137	98.4	5	9	13	14	16	154	158	4.16	0.98
28C6d3	3	8,911	5,439	158	98.1	6	9	15	16	11	206	197	4.11	0.97
28C6d4	4	4,264	3,149	156	96.8	8	10	20	21	27	176	179	4.15	0.97
28C6d5	5	4,869	2,325	145	95.6	9	12	16	22	29	161	158	4.08	0.97
28C6d6	6	9,043	6,169	108	98.7	4	7	13	15	19	145	138	3.61	0.95
Culture-dependent samples														
28C6d128[a]	1 – 6	12,306	10,051	140	99.1	2	3	6	4	11	155	159	2.56	0.85
34 °C day 6														
Culture-independent samples														
34C6d1	1	3,810	2,678	108	97.6	3	6	11	13	18	121	128	3.62	0.96
34C6d2	2	10,382	8,576	156	98.5	5	11	20	16	26	225	193	3.92	0.96
34C6d3	3	10,046	5,464	77	98.6	5	9	15	18	21	104	107	1.73	0.61
34C6d4	4	9,007	5,516	112	98.0	5	9	13	14	7	144	136	3.71	0.95
34C6d5	5	8,872	5,524	112	98.2	6	9	12	11	10	132	137	3.58	0.95
34C6d6	6	5,092	3,107	133	97.1	7	11	20	22	23	151	151	3.84	0.96
Culture-dependent samples														
34C6d178[a]	1 - 6	12,012	9,064	197	99.1	2	4	6	7	14	203	205	3.66	0.94

[a] Pool of all colonies cultured on marine agar.

3.4.2.2 Alpha diversity analyses

Rarefaction curves are in Figure 3-3. The dataset was subsampled to a depth of 2,325 – 10,051 (Table 3-1). The observed operational taxonomic units (OTUs) for the haemolymph of control lobsters (28 °C) ranged from 108 to 172 on 6 dpt (Table 3-1). The haemolymph of animals exposed to 34 °C for 6 d had 77 – 156 observed OTUs. There were no significant differences in the observed OTUs (t_{10} = 2.106, P = 0.061), richness estimators (Chao1 [t_{10} = 1.366, P = 0.202], ACE [t_{10} = 1.930, P = 0.082]), or diversity indices (Shannon [U = 6.000, P = 0.065], Simpson [U = 9.000, P = 0.158) between juveniles held at either temperature for 6 d (Table 3-1). The culture-dependent samples exhibited very high OTU richness that was dominated by only a few OTUs.

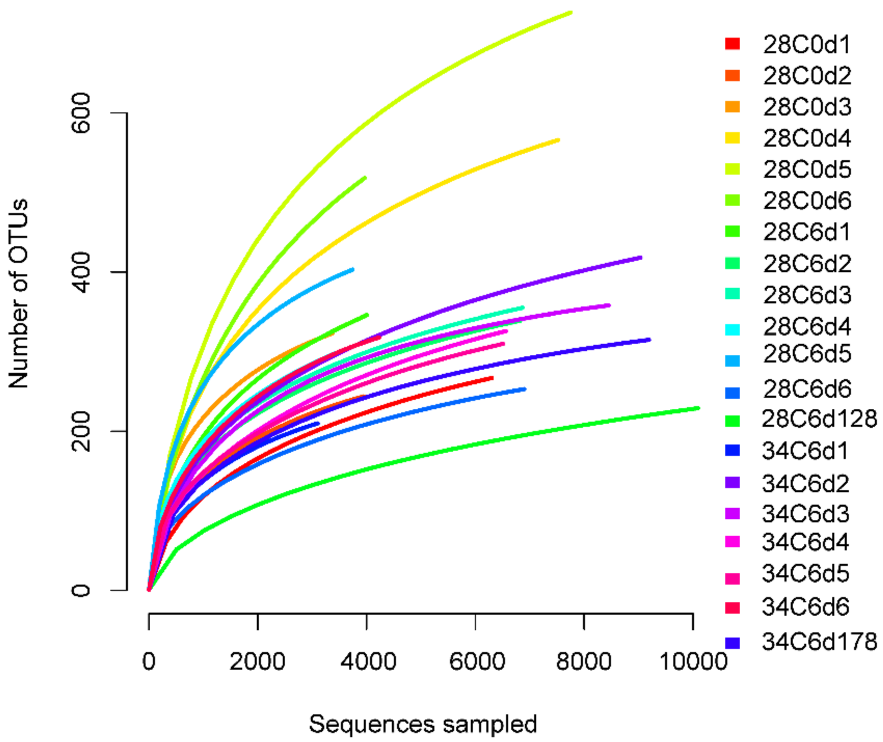


Figure 3-3. Rarefaction curves for haemolymph sequence libraries of *P. ornatus* juveniles (see Table 3-1 for abbreviation of samples).

3.4.2.3 Beta diversity analyses

The first two axes of principal coordinate analyses (PCoA) based on Bray Curtis, weighted UniFrac and unweighted UniFrac distance matrices explained 27.3, 38.0 and 24.7% of the variation in abundance of OTUs among different sample libraries, respectively (Figure 3-4). This variation was not related to treatment groups except for the unweighted UniFrac where there was some separation along the second axis. The PCoA results were supported by PERMANOVA. When the Bray Curtis index (R^2 = 0.143, P = 0.083), weighted UniFrac (R^2 = 0.123, P = 0.357) and unweighted UniFrac (R^2 = 0.150, P = 0.031) distance matrices were analysed statistically using PERMANOVA, only the last model showed significant difference among the three types of culture-independent haemolymph libraries.

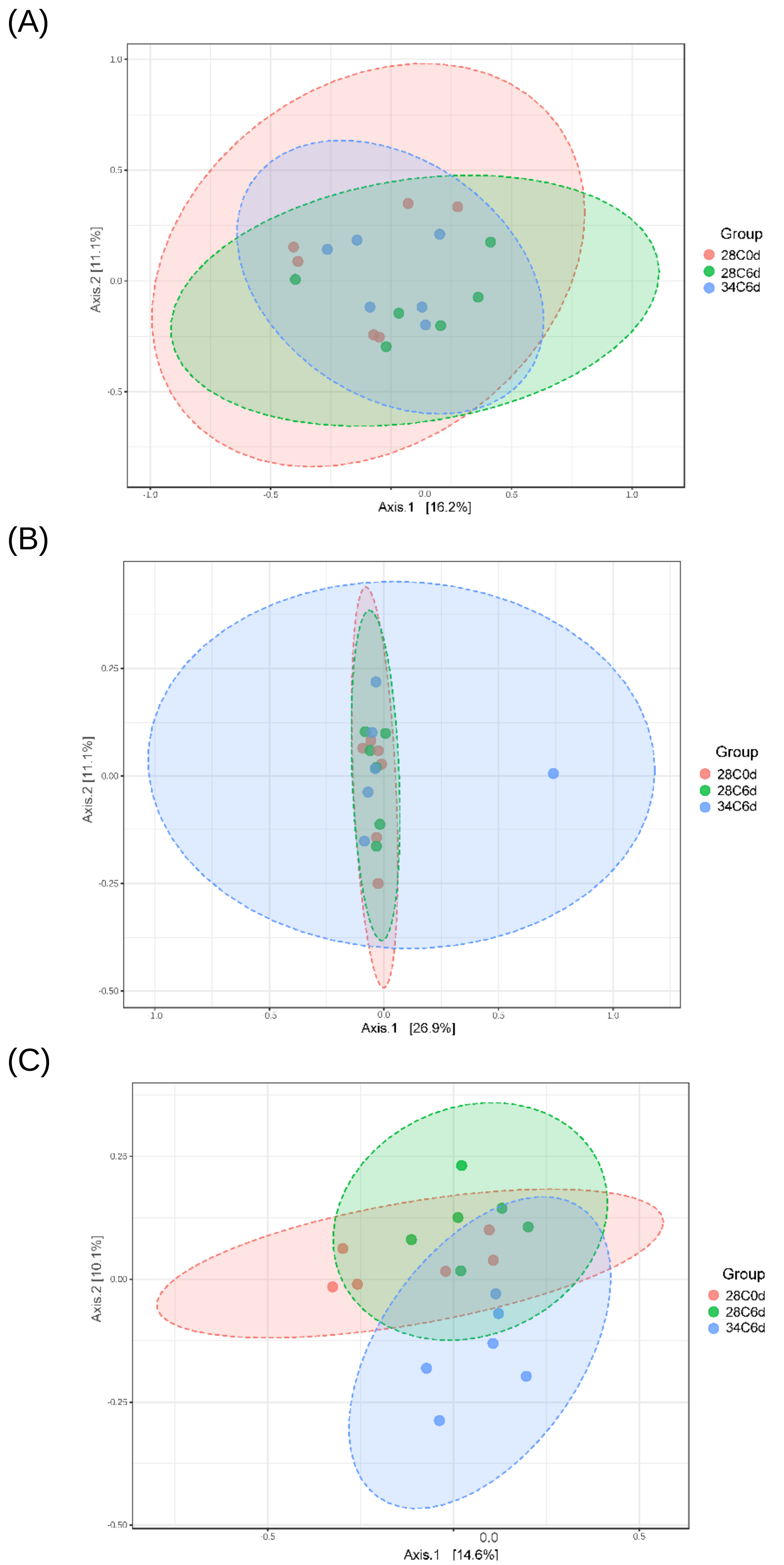


Figure 3-4. Principal coordinate analysis plots based on (A) Bray Curtis index, (B) weighted UniFrac and (C) unweighted UniFrac distance methods showing similarity in haemolymph sequence libraries of *P. ornatus* juveniles. Key: 28C0d: 28 °C 0 dpt; 28C6d: 28 °C 6 dpt; 34C6d: 34 °C 6 dpt.

3.4.2.4 Taxonomic composition

3.4.2.4.1 Culture-independent samples

Sequencing analysis of culture-independent haemolymph sample libraries indicated that the core microbiome of juvenile *P. ornatus* consisted of Proteobacteria and Bacteroidetes (Figure 3-5A). This was based on highly abundant and prevalent OTUs grouped by phylum and excluded bacterial taxa not assigned (i.e. OTUs with 97% nucleotide sequence identity that were unable to be assigned to known bacterial taxa in the Greengenes database with a confidence threshold of 0.8 using RDP Bayesian Classifier). The Venn diagram (Figure 3-5B) showed that the three groups of haemolymph samples shared 327 OTUs (11% of total OTUs) which included genera *Ruegeria*, *Acinetobacter*, *Pseudomonas*, *Loktanella*, *Cohaesibacter*, *Psychrobacter*, BD2_13, *Nautella*, *Phaeobacter*, *Thalassobius* and *Antarcticimonas*. The haemolymph of control lobsters on 6 dpt shared significantly more OTUs (χ^2 = 8.377, P = 0.004) with those on 0 dpt (293 OTUs; 22% of 28C6d) than thermally challenged juveniles on 6 dpt (227 OTUs; 17% of 28C6d). Control animals on 0 dpt had the highest proportion of unique OTUs (752; 46% of 28C0d).

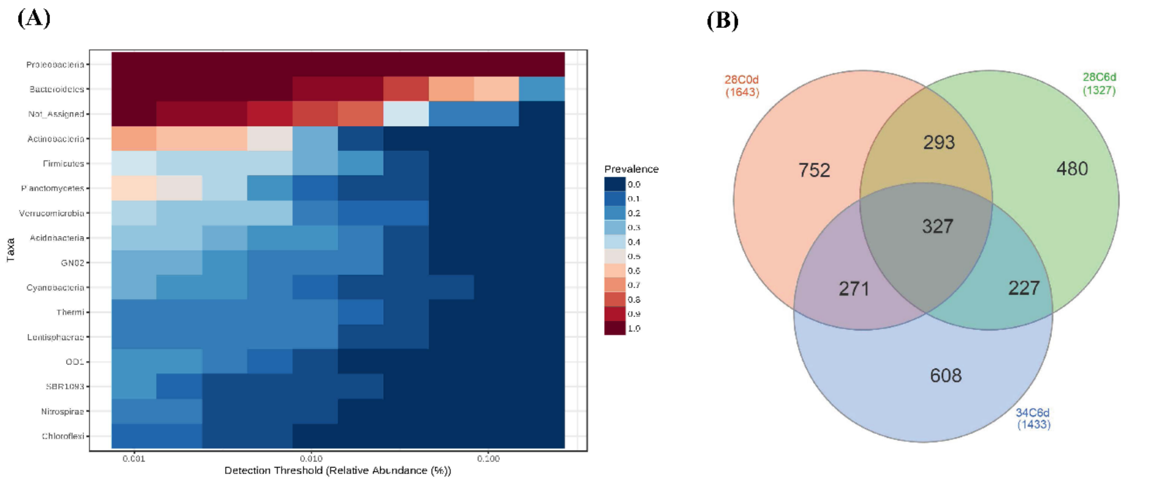


Figure 3-5. (A) Core microbiome analysis based on relative abundance and prevalence of bacterial OTUs grouped by phyla from haemolymph samples of juvenile *P. ornatus*. (B) Venn diagram showing shared and unique OTUs in haemolymph samples of juvenile *P. ornatus.* Key: 28C0d: 28 °C 0 dpt; 28C6d: 28 °C 6 dpt; 34C6d: 34 °C 6 dpt.

The composition of haemolymph bacterial communities of control and stressed animals at day 6 were analysed using the MetagenomeSeq statistical pipeline at phylum, class, family and genus levels. The culture-independent haemolymph libraries of lobster groups (28 °C 0 dpt, 28 °C 6 dpt, 34 °C 6 dpt) were mostly dominated by Proteobacteria and Bacteroidetes (Figure 3-6A). Phyla Verrucomicrobia (P = 0.046) and Thermi (P = 0.018) were significantly more represented in the haemolymph of control lobsters (Verrucomicrobia 1.7 ± 0.9%, Thermi 0.7 ± 0.5%) than that of thermally stressed animals on 6 dpt. The four major classes represented in the haemolymph libraries of juvenile lobsters were Alphaproteobacteria, Gammaproteobacteria, Saprospirae and Flavobacteriia (Figure 3-6B). The haemolymph of animals exposed to 28 °C had significantly higher abundance of classes Verrucomicrobiae (1.6 ± 1.0%; P < 0.001), Deinococci (0.7 ±

0.5%; P < 0.001), Acidimicrobiia (0.4 ± 0.3%; P = 0.003), Clostridia (0.9 ± 0.6%; P = 0.024) and Lentisphaeria (1.0 ± 0.7%; P = 0.032) than that of juveniles exposed to 34 °C on 6 dpt. The most abundant bacterial families found in the haemolymph were *Rhodobacteraceae*, *Saprospiraceae*, *Flavobacteriaceae* and *Cohaesibacteraceae* (Figure 3-6C). Families *Trueperaceae* (0.7 ± 0.5%; P = 0.020), *Kordiimonadaceae* (2.5 ± 1.7%; P = 0.039), *Bradyrhizobiaceae* (0.7 ± 0.5%; P = 0.043) and *Acidaminobacteraceae* (0.9 ± 0.6%; P = 0.045) were significantly more represented in the haemolymph of control juveniles compared to that of thermally stressed lobsters on 6 dpt. The predominating bacterial genera were *Ruegeria*, *Acinetobacter*, KD1_23, *Pseudomonas* and *Loktanella* (Figure 3-6D). On 6 dpt, the haemolymph of 28 °C exposed juveniles had significantly higher abundance of *Loktanella* (4.2 ± 3.2%; P = 0.005), *Cohaesibacter* (3.8 ± 2.4%; P = 0.006), *Polaribacter* (0.6 ± 0.3%; P = 0.028) and *Micrococcus* (0.2 ± 0.2%; P = 0.028) than 34 °C exposed lobsters. Overall, the abundance of rare haemolymph OTUs at all taxonomic levels decreased in thermally stressed animals.

3.4.2.4.2 Culture-dependent samples

Haemolymph sequence libraries comprising pooled isolates cultured from lobsters either exposed to control or elevated temperature at day 6 were represented only by phyla Proteobacteria and Bacteroidetes (Figure 3-6A). The two predominating classes of culturable bacteria belonged to the Alphaproteobacteria and Gammaproteobacteria (Figure 3-6B). There were fewer families represented in the haemolymph libraries of culturable bacteria when compared to culture-independent bacteria, with the former dominated by *Rhodobacteraceae* and *Moraxellaceae* (Figure 3-6C). The most represented culturable bacterial genera in the haemolymph samples were *Ruegeria*, *Psychrobacter*, *Phaeobacter* and *Nautella* (Figure 3-6D). Genera *Acinetobacter*, *Pseudomonas* and BD2_13 were not represented in culture-dependent samples but featured in culture-independent sample libraries (Figure 3-6D).

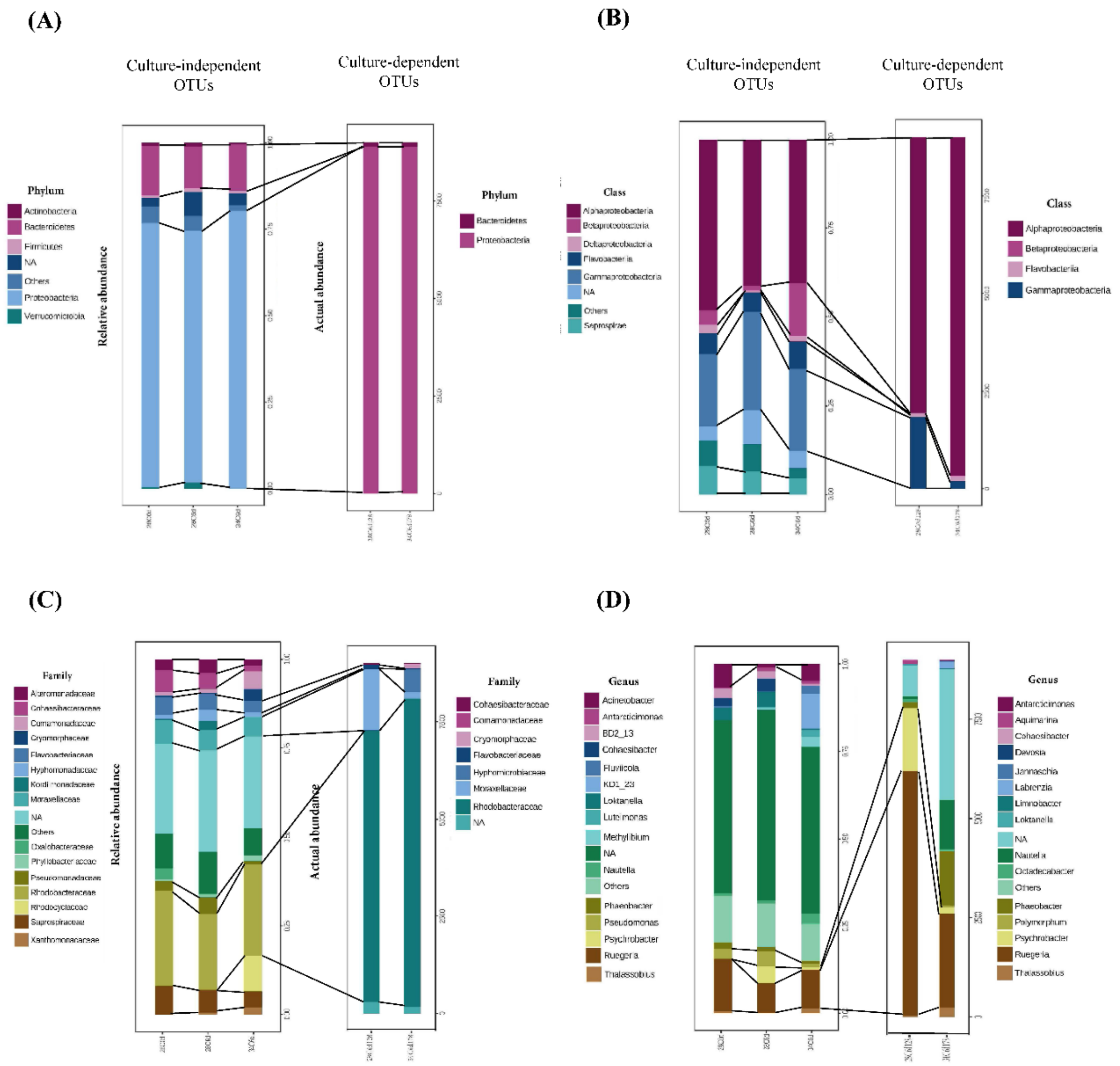


Figure 3-6. Comparisons of relative abundance (culture-independent) and actual abundance (culture-dependent) of OTUs in haemolymph sample libraries of juvenile *P. ornatus* at (A) phylum, (B) class, (C) family and (D) genus levels. Key: 28C0d: 28 °C 0 dpt; 28C6d: 28 °C 6 dpt; 34C6d: 34 °C 6 dpt; 28C6d128: cultured colonies from 28 °C 6 dpt; 34C6d178: cultured colonies from 34 °C 6 dpt; NA: not assigned to bacterial taxa based on classification methods used in this study.

3.4.2.5 Functional potential

PICRUSt was employed to computationally predict gene families found in lobster haemolymph communities and metabolic functional profiles were then assigned using KEGG. Amino acid metabolism, metabolism of other amino acids and metabolism of cofactors and vitamins were identified as the main metabolic functions of lobster haemolymph communities (Figure 3-7). Three KEGG pathways were significantly over represented in the haemolymph of control lobsters when compared to animals exposed to 34 °C on 6 dpt: sphingolipid metabolism ($P = 0.020$; K00720, K01201, K01190; lipid metabolism), phosphonate and phosphinate metabolism ($P = 0.031$; K01841, K05306; metabolism of other amino acids) and other glycan degradation ($P = 0.044$; K01190, K12111, K12112, K12373, K01201; glycan biosynthesis and metabolism).

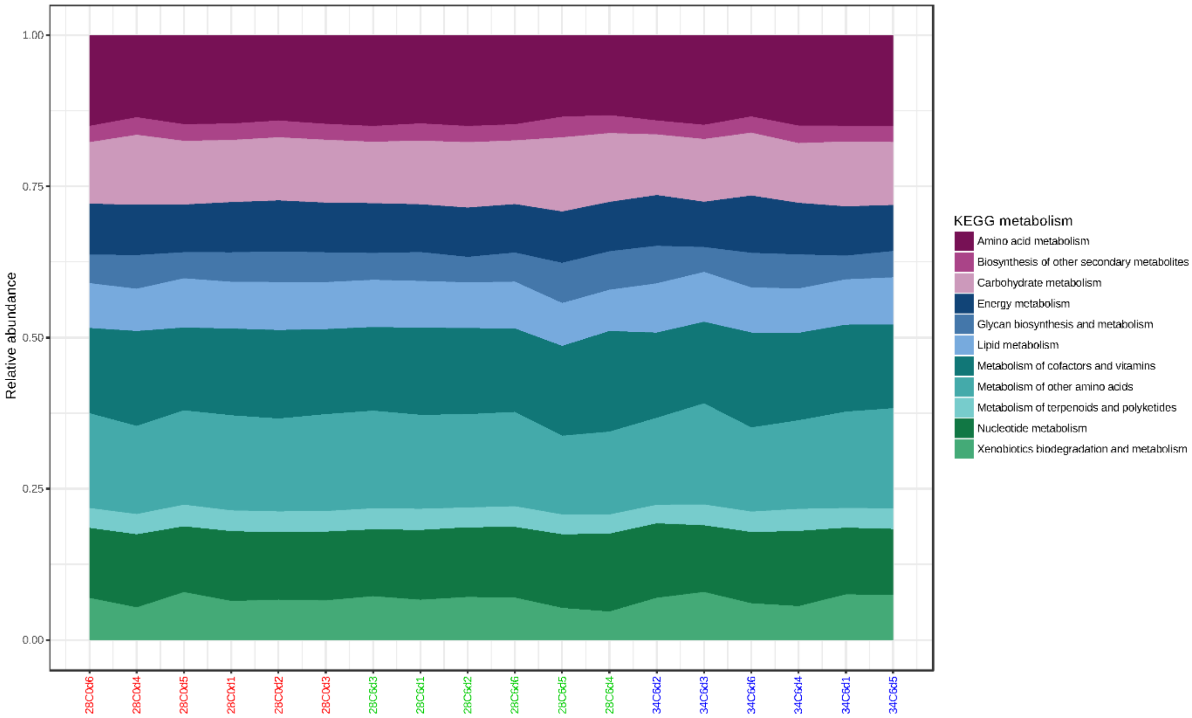


Figure 3-7. Functional diversity profiling of culture-independent OTUs in haemolymph samples of juvenile *P. ornatus* based on KEGG metabolism using PICRUSt. Key: 28C0d: 28 °C 0 dpt; 28C6d: 28 °C 6 dpt; 34C6d: 34 °C 6 dpt.

3.4.3 Bacterial load

The concentrations of bacteria among lobsters ranged from 0 to 3.19×10^3 CFU mL^{-1} (culturable bacteria) and from 55 to 1.31×10^4 cell equivalents mL^{-1} (*rpoB* gene, qPCR). As revealed by qPCR, bacterial loads were significantly different ($U = 2.000$, $P = 0.009$) only between thermally stressed lobsters and control animals at 4 dpt (Figure 3-8). There was no significant difference ($P > 0.05$) in the culturable bacterial load between animals exposed to 34 °C and 28 °C through time.

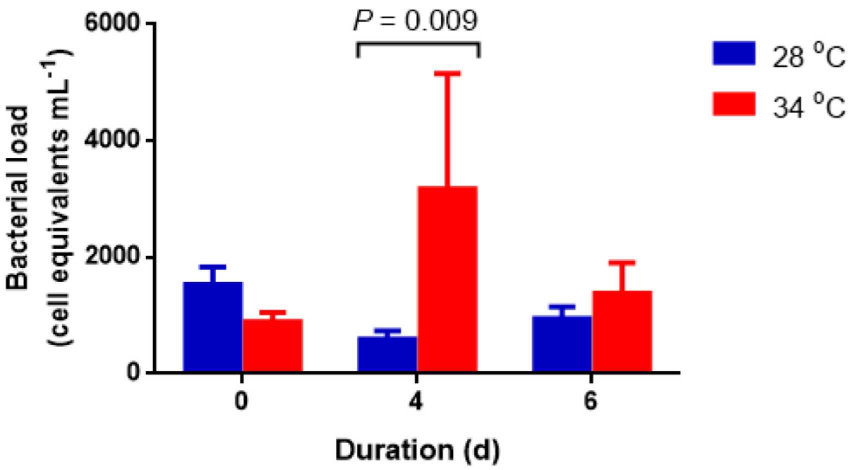


Figure 3-8. Bacterial load in the haemolymph of *P. ornatus* juveniles exposed to 28 °C and 34 °C based on culture-independent (*rpoB* gene) technique. Each bar is mean + SEM, $n = 6$.

3.5 Discussion

3.5.1 Overview

This is the first study to comprehensively characterise and quantify the haemolymph microbiome of juvenile *P. ornatus*. We found that exposure to increased temperature reduced survival rate and affected haemolymph immune response (total granulocyte counts), the diversity, load (culture-independent method) and functional profile of haemolymph bacterial communities. Culture-independent bacterial community analyses captured a higher bacterial diversity and load when compared to the culture-dependent method, however both indicated phyla Proteobacteria and Bacteroidetes were predominating members of the haemolymph microbiome. We also showed that bacterial load of the haemolymph was understated using culture based plating methods when compared to qPCR, consistent with findings of those of Givens et al. (2013) in analysis of haemolymph from crab *C. sapidus*. Our observed changes to haemolymph bacterial load and diversity of *P. ornatus* are consistent with other temperature stress studies on aquatic invertebrates including the Pacific oyster *Crassostrea gigas* (Lokmer and Wegner, 2015), crawfish *P. clarkii* (Scott and Thune, 1986) and crab *C. sapidus* (Tubiash et al., 1975).

3.5.2 Survival and immune parameters

In the present study, lobster mortality occurred after 4 days of increased temperature, and was associated with signs of lethargy and opaque muscle. As optimal growth temperatures established for *P. ornatus* are in the range of 25 to 31 °C (Jones, 2009), normal metabolism becomes compromised at higher temperatures and exposure to 34 °C may be approaching the physiological thermal limit. These observations were concomitant with a significant decrease in total granulocyte counts, which could have decreased the capacity to protect against opportunistic bacteria and leave the animal susceptible to infection (Evans, 2003). Granulocytes are responsible for releasing phenoloxidase (Johansson and Soderhall, 1989), which activates the encapsulation of pathogens and tissue repair mechanisms (tissue scaffolding) in invertebrates. This could explain the increase in total granulocyte counts as a response to the higher bacterial load on 4 dpt, which lead to the decrease in both parameters on 6 dpt in this study. Phenoloxidase activity was found to decrease in response to increased temperatures in Pacific white shrimp *Litopenaeus vannamei* (Cheng et al., 2005) and yellowleg shrimp *Penaeus californiensis* (Vargas-Albores et al., 1998). Unlike total granulocyte counts, total haemocyte counts (THC) did not differ significantly between control and 34 °C animals in the present study. Studies have shown that acute thermal stress ($\leq$ 7 dpt) resulted in significant decreases in THC of Mediterranean shore crab *Carcinus aestuarii* (Matozzo et al., 2011) and shrimp *L. vannamei* (Cheng et al., 2005), whereas chronic thermal stress (> 14 dpt) can increase THC in lobster *H. americanus* (Dove et al., 2005).

The thermal increment of 6 °C greater than the best practice culture temperatures did not have a profound effect on THC or clotting times in the haemolymph of *P. ornatus*. This could be due to the acute exposure time-frame used in the experiment (6 d). Previous studies have shown that changes to THC of thermally stressed lobster *H. americanus* (Dove et al., 2005) and crab *C. aestuarii* (Matozzo et al., 2011) were not observed until 7 dpt. Other possible reasons for the apparent lack of temperature effect on these parameters are the tight modulation of haemolymph by the host and associated microbiota. For example, cellular (i.e. haemocyte proliferation) and immune (i.e. increased phenoloxidase activity) parameters in the haemolymph of crab *C. aestuarii* were host regulated with temperature changes (Matozzo et al., 2011).

3.5.3 Bacterial diversity

The core microbiome of juvenile *P. ornatus* haemolymph consisted of Proteobacteria (Alpha-, Gamma-) and Bacteroidetes irrespective of thermal exposure treatment. Similarly, the majority of the bacteria cultured from the haemolymph of apparently healthy lobster species including *P. ornatus* (Evans et al., 2003), *P. cygnus* (Evans et al., 1996), *P. homarus* (Mary Leema et al., 2010), *J. edwardsii* (Handlinger et al., 2006) and *H. americanus* (Bartlett et al., 2008) belonged to Proteobacteria. Proteobacteria and / or Bacteroidetes were also the dominant phyla of bacterial communities in the circulatory system of other aquatic invertebrates, such as the Pacific oyster *C. gigas* (Lokmer and Wegner, 2015) and blue crab *C. sapidus* (Givens et al., 2013). This may suggest the importance of these phyla in the host or reflect their ubiquitous distribution in the environment. Furthermore, as bacteria may be transmitted from the digestive tract to the circulatory system, the gut microbiome may be related to the haemolymph microbiome (Davis and Sizemore, 1982). Our research group has recently shown that the core gut microbiome of juvenile *P. ornatus* cultured from the same facility shared Proteobacteria with the core haemolymph microbiome in addition to Tenericutes (Ooi et al., 2017). Bacterial families that were found in both the gut and haemolymph include *Flavobacteriaceae*, *Saprospiraceae*, *Rhodobacteraceae*, and *Moraxellaceae*.

Rhodobacteraceae were detected in all culture-dependent and culture-independent samples. *Rhodobacteraceae*, and in particular the genus *Ruegeria*, was predominant in an earlier culture-based study on bacteria in the haemolymph of *P. ornatus* juveniles from the same facility (unpub. data). *Ruegeria* commonly form symbiotic relationships with aquatic animals by producing antimicrobials and are major contributors of carbon and sulphur cycles in the marine environment (Brinkhoff et al., 2004; Wagner-Döbler and Biebl, 2006; Bruhn et al., 2007; Pujalte et al., 2014). In contrast, other members of *Rhodobacteraceae* such as *Nautella* also detected across haemolymph libraries in our study, are known marine pathogens implicated in algal bleaching (Fernandes et al., 2011). Furthermore, Stratil et al. (2013) reported an increase in *Rhodobacteraceae* abundance in brown macroalga *Fucus vesiculosus* with increasing temperature, which may be associated with higher disease risk. Conversely, in our study the abundance of a haemolymph-associated *Rhodobacteraceae* genus (i.e. *Loktanella*) decreased under thermal stress.

Bacterial taxa that were characterised consistently by culture-dependent and culture-independent methods across all the experimental treatments in this study are likely to have important haemolymph functions or associations with *P. ornatus*. The persistence of *Cohaesibacteraceae*, *Flavobacteriaceae*, and *Moraxellaceae* in the haemolymph across all treatments suggests that these genera are resident members (commensals or symbionts) as opposed to transient populations (Harris, 1993) of the haemolymph microbiome. Members of *Cohaesibacteraceae* including *Cohaesibacter* are known nitrate reducers, capable of converting nitrate to nitrite that may then be utilised by host nitric oxide synthases to generate nitric oxide (Gallego et al., 2010; Qu et al., 2011; Rathod et al., 2016). This could be important for the immune response of lobsters as nitric oxide is involved in the antimicrobial defences of crustaceans (Li et al., 2016; Rodríguez-Ramos et al., 2016). *Flavobacteriaceae* have been reported to have symbiotic relationships with insects and produce enzymes that can degrade organic compounds (Bernardet and Nakagawa, 2006). *Psychrobacter* of family *Moraxellaceae* have been described to have antagonistic effects against pathogenic bacteria (Sun et al., 2009; Yang et al., 2011), lipolytic activity and the ability to reduce nitrate to nitrite (Romanenko et al., 2008). The persistence of *Psychrobacter* has been reported previously in the haemolymph of Dungeness crab *Cancer magister* (Scholnick and Haynes, 2012). On the other hand, Chistoserdov et al. (2005) and Baross et al. (1978) have linked the presence of *Flavobacteriaceae* and *Moraxellaceae* with disease in crustaceans, although there was no suggestion this was the case in our study.

The changes in haemolymph bacterial abundance from lobsters sampled on 6 dpt could indicate thermal stress had a dysbiotic effect on the communities. The majority of haemolymph OTUs impacted by elevated temperature have been reported in the marine environment such as *Kordiimonadaceae* (Ghosh and Bhadury, 2017), *Acidaminobacteraceae* (Koo et al., 2015), *Loktanella* (Yoon et al., 2007), and *Polaribacter* (Nedashkovskaya et al., 2005). *Micrococcus* are known symbionts in marine sponges (Lee et al., 2001). In most cases the relative abundance of the aforementioned lobster haemolymph OTUs were reduced or had disappeared at elevated temperature, that could infer significant function loss. Further investigation of these bacterial groups would be required as their decline or absence could indicate stress.

The predicted functions of bacterial communities generated by the PICRUSt algorithm may assist in explaining the role of bacteria in the haemolymph. In the present study, the main putative functions of haemolymph communities were likely to be metabolism of amino acid and other amino acids, that may be associated with amino acids and non-protein amino acids in the haemolymph (Maynard, 1960). Amino acid metabolism is also a major function of the global ocean microbiome (Sunagawa et al., 2015), which implies that bacterial-produced amino acids are crucial to oceanic trophic interactions. Phosphonate and phosphinate metabolisms were also affected by temperature, similar to the results of a salinity stress study on shrimp *L. vannamei* (Chen et al., 2015a). Phosphonate and phosphinate metabolism can only be conducted by protists and bacteria, particularly Actinobacteria (Kanehisa et al., 2016) which were found in *P. ornatus* haemolymph in this study. The third most abundant metabolic function involved vitamins and cofactors, which may not be synthesised or synthesised in limited quantities by lobsters (Takeuchi and Murakami, 2007) rendering them dependent on bacteria in the haemolymph. Other KEGG pathways that were influenced by elevated temperature were glycan degradation (other) and sphingolipid metabolism. Both pathways are part of glycosphingolipid biosynthesis (Kanehisa et al., 2016). Sphingolipids in some bacteria and fungi may be involved in attachment to host or microbial cells (Olsen and Jantzen, 2001), and signalling during heat stress (Skrzypek et al., 1999). Functional changes of indigenous bacterial communities could be a driver in reducing host health in the present study, where specific bacterial metabolic pathways are diminished under certain conditions such as thermal stress.

3.5.4 Implications

Non-lethal sampling of haemolymph is an important consideration when assessing asymptomatic bacteraemia in both healthy and diseased animals for effective health evaluations. For spiny lobsters (e.g., *Panulirus interruptus*), haemolymph constitutes about 30% of the total animal wet weight (Belman, 1975). Radford et al. (2005) demonstrated previously that lobster *J. edwardsii* juveniles make a full recovery within 3 weeks following removal of 10 – 15% of haemolymph volume. Moreover, several studies that conducted repeat sampling from individual crustaceans (Butler et al., 1978; Hagerman, 1983; Chang et al., 1999; Evans, 2003; Radford et al., 2005; Scholnick and Haynes, 2012) and Evans (2003) found no decrease in protein level. In the present study, lobsters were euthanised due to the large volume of haemolymph required for several immune and bacterial assays. Only half a millilitre of haemolymph was used for culture-independent bacterial analyses, representing ~ 1% of the total haemolymph volume of the average weight of juveniles. Non-lethal sampling of such volumes of haemolymph would allow for animal recovery and provide a feasible means to conduct ongoing lobster health monitoring programs.

3.5.5 Limitations

In this study, all cultured haemolymph bacteria were cultivated at 28 °C despite some communities being derived from lobsters exposed to 34 °C. We recognise this may have introduced a selective force bias, however this could have been offset by the growth of bacteria at 28 °C that would otherwise be in stressed or viable but non-culturable states when cultivated at 34 °C. This is supported in part by our existing analyses that still revealed differences in bacterial diversity between the culturable haemolymph communities of lobsters exposed to 28 °C and 34 °C although this could not be statistically validated due to the small (pooled) sample size. The nested PCR approach used for the culture-independent analyses of our samples may have also produced confounding results. It is generally accepted that next-generation amplicon sequencing read abundances are biased to varying degrees by 16S copy number, PCR primer mismatches and the number of PCR cycles (Edgar, 2017). 16S amplicon abundance bias remains unavoidable and as such we minimised the number of PCR cycles and therefore the potential for bias in our nested PCR. This approach was suggested previously by Yu et al. (Yu et al., 2015) who proposed that nested PCR is acceptable when standard PCR cannot be used. Although the functional diversity profile of OTUs may be affected by the variation of 16S rRNA copy numbers in different bacteria (Langille et al., 2013), the functional association analysis was compared between bacteria in the stressed and control lobsters which should have limited the copy number variations.

3.5.6 Conclusion

This is the first study to characterise and quantify haemolymph responses and the microbiome of *P. ornatus* exposed to thermal challenge. Increased temperature (34 °C) affected survival, total granulocyte counts, bacterial diversity, bacterial load and changed the predicted functional profile of haemolymph bacterial communities. The core microbiome of the haemolymph as revealed by both culture-dependent and culture-independent analyses was represented by Proteobacteria (Alpha-, Gamma-) and Bacteroidetes. *Rhodobacteraceae* (Alphaproteobacteria) were prevalent across all samples. This study shows that non-lethal sampling and the subsequent examination of changes to the haemolymph microbiome has potential for health monitoring programs for spiny lobsters.

Chapter 4 Developmental and gut-related changes to microbiomes of the cultured juvenile spiny lobster *Panulirus ornatus*

Part of the research contained within this chapter has been published as Ooi, M.C., Goulden, E.F., Smith, G.G., Nowak, B.F., Bridle, A.R., 2017. Developmental and gut-related changes to the microbiomes of the cultured juvenile spiny lobster *Panulirus ornatus*. FEMS Microbiol. Ecol. 93, fix159.

4.1 Abstract

With recent technologies making it possible for closed life cycle aquaculture production of spiny lobster (*P. ornatus*) at commercial scale comes a strong impetus to further understand aspects of lobster health. The gut microbiome plays a crucial role in host health, affecting growth, digestion, immune responses and pathogen resistance. Herein we characterise and compare gut microbiomes across different developmental stages (6 – 7 d post-emergence [dpe], 52 dpe and 13 months post-emergence [mpe]) and gut regions (foregut, midgut and hindgut) of cultured *P. ornatus* juveniles. Gut samples were analysed using 16S rRNA next-generation sequencing. Core gut microbiomes of *P. ornatus* comprised the phyla Tenericutes and Proteobacteria. Within class Gammaproteobacteria, families *Pseudoalteromonadaceae* and *Vibrionaceae* were dominant members across the majority of the gut microbiomes. Characterisation of bacterial communities from 13 mpe lobsters indicated that the hindgut microbiome was more diverse and compositionally dissimilar to the fore- and midgut. The bacterial composition of the hindgut was more similar among younger juveniles (6 - 7 dpe and 52 dpe) compared to 13 mpe lobsters. This is the first study to explore gut microbiomes of spiny lobster

juveniles. We demonstrate that the composition of the gut microbiome was shaped by gut region, whereas the structure of the hindgut microbiome was influenced by developmental stage.

4.2 Introduction

The ornate spiny lobster, *P. ornatus* is widely distributed over the Indo-West Pacific region where it supports important artisanal and commercial fisheries. Like many wild spiny lobster stocks throughout the world this species is vulnerable to increased fishing pressure and climate change (Plagányi et al., 2011; Norman-López et al., 2013). Recently, breakthrough developments in spiny lobster hatchery systems and nutrition by University of Tasmania-Nexus Aquasciences Pty Ltd (UNA) have made it possible for commercial scale closed life cycle production. It is expected that the development of spiny lobster aquaculture based on hatchery reared seed stock will reduce the wild harvest of lobster seed stock, alleviate fishing pressures by supplementing food production chains, and contribute to stock enhancement programs. This technology will enable a sustainable and reliable supply of stock and has the potential to improve the socioeconomic status of communities that are dependent on spiny lobster fisheries for their livelihoods. The UNA culture technologies are most advanced for *P. ornatus*, which is an attractive aquaculture species due to its fast growth and high price in Asian markets (Jeffs, 2010). However, a number of challenges persist during larviculture and grow-out of juveniles including bacterial diseases demanding greater efforts to improve the health and survival of cultured lobsters (Jones, 2015).

The microbiota closely associated with organisms is a growing and fruitful field of research. The largest undertaking has been the human microbiome project (HMP) that akin to the human genome project characterised the microbiota associated with diseased and healthy humans. These microbiota have been found to play a crucial role in maintaining human health by enhancing the immune response, facilitating growth and nutrition, and by increasing pathogen resistance (Cho and Blaser, 2012). This area of research has led to the development of tools and a conceptual understanding of the composition and function of the gut microbiomes of several aquaculture species (Llewellyn et al., 2014). Next-generation sequencing (NGS) techniques have substantially improved our knowledge of gut bacterial communities associated with varying health states and in response to diets and the environment (Ghanbari et al., 2015). Several studies have employed NGS to describe the complex gut bacterial communities of crustaceans, including the Norway lobster *N. norvegicus* (Meziti and Kormas, 2013), cherry shrimp *N. denticulata* (Cheung et al., 2015), Pacific white shrimp *L. vannamei* (Huang et al., 2014; Zhang et al., 2014), black tiger shrimp *P. monodon* (Rungrassamee et al., 2013) and giant freshwater prawn *M. rosenbergii* (Mente et al., 2016).

Although the host and external factors may affect the composition of the gut microbiome (Spor et al., 2011), less is known regarding the microbiota of different gut regions and how the developmental stage influences the microbial communities. The digestive tract of crustaceans is divided into three regions: the foregut, midgut (including midgut gland) and hindgut (Ceccaldi, 1989). In brief, ingested food is mechanically ground in the foregut before moving to the midgut. The processes of nutrient solubilisation by digestive enzymes, nutrient absorption, and removal of fluids from indigestible particles and their coating with a peritrophic membrane occurs in the midgut (Perera and Simon, 2015). The faecal pellet is then moved into the hindgut for excretion (Perera and Simon, 2015). Although there are region-specific functions in the crustacean gut, Cheung et al. (2015) did not find distinct compositional differences in microbiota between the gut regions of *N. denticulata*, and reported the consistent presence of members of the phylum Proteobacteria. Physiological and immunological changes that occur during development may also impact the microbial diversity of the gut (Rungrassamee et al., 2013; Cheung et al., 2015). Nonetheless, Huang et al. (2014) and Rungrassamee et al. (2013) showed that the same bacterial phyla were predominant in the gut of *L. vannamei* at all developmental stages. External factors such as feed and water quality have varied effects on gut bacterial communities of different species, with a strong influence reported for *L. vannamei* (Qiao et al., 2016) and little impact for *N. norvegicus* (Meziti et al., 2012) and *L. vannamei* (Zhang et al., 2014).

Given the importance of gut-associated bacteria to the overall health of an organism, the aim of this study was to characterise and compare the gut microbiomes across different developmental stages and regions of the digestive tract (fore-, mid- and hindgut) of cultured *P. ornatus* juveniles.

4.3 Materials and methods

4.3.1 Juvenile lobster culture system

Panulirus ornatus were produced from embryos that hatched as larvae and metamorphosed into juveniles in two batches (1 and 2) to allow comparisons of different developmental stages that span several months. Juveniles were sired from the same male broodstock. Lobsters were produced at the Institute for Marine and Antarctic Studies (IMAS), in Hobart, Australia according to protocols modified from Jensen et al. (2013) and Fitzgibbon and Battaglene (2012). For batch 1, newly emerged juveniles (J1) were held in either individual or communal holding systems. Individual systems housed J1s (first juvenile instar) to 7 d post-emergence (dpe) and were comprised of 8 L containers segregated into 8 individual compartments and supplied with flow-through water (temperature 27.72 ± 0.04 ^{o}C; dissolved oxygen [DO] 105.30 ± 0.66 %; pH 8.19 ± 0.01; salinity 33.57 ± 0.15 g L^{-1}; and flow-rate 6.0 exchanges h^{-1}). For communal holding, juveniles were cultured to 52 dpe in floating rectangular mesh cages (surface area: 1.17 m^2) housed within a 4000 L culture vessel on a recirculating water flow system (temperature 27.99 ± 0.15 ^{o}C; DO 102.55 ± 0.59 %; pH 8.13 ± 0.02 and salinity 33.29 ± 0.02 ppt). Animals held individually were fed once per day with fresh blue mussel (*Mytilus galloprovincialis*) mantle and gonad, and twice per day with a commercial Kuruma prawn pellet (Higashimaru, Vital No. 12, http://www.k-higashimaru.co.jp/). Communal animals were fed Kuruma prawn pellet four times daily rationed at a rate of approximately 10 % total tank biomass. All juveniles were provided with cylindrical hides and cultured in predominate darkness. Three 6 - 7 dpe J1s (0.14 ± 0.001 g) and three 52 dpe juveniles (1.27 ± 0.12 g) were sampled. All sampled juveniles were apparently healthy and in intermoult phase.

For batch 2, juveniles were held communally to 13 months post-emergence (mpe) in 600 L fibreglass vessels supplied with flow-through seawater (temperature 26.44 ± 0.08 ^{o}C; DO 100.01 ± 0.47 %; pH 7.88 ± 0.01; salinity 34.50 ± 0.12 ppt; and flow-rate 0.36 exchanges h^{-1}). Juveniles were fed slightly over-satiation a combination of fresh blue mussels and Kuruma prawn pellet daily. Animals were cultured under 12:12 h light:dark photoperiod and provided with Z-stack hides for shelter. Three juveniles (209.3 ± 10.3 g) were sampled at 13 months of culture. Juveniles were starved for 24 h prior to sampling.

4.3.2 Sample collection

All lobster dissections and sampling were performed using aseptic techniques. Surgical tools were sterilised in 1:20 chlorine 4 % w/v, 70 % ethanol and flamed prior to use and between incisions. The 6 - 7 and 52 dpe juveniles were euthanised in a seawater ice slurry for approximately 1 min, surface sterilised by dipping in 70 % ethanol for 15 s and placed on sterile Petri dishes for dissections. The hindgut was extracted from the anterior region of the abdomen following detachment from the cephalothorax and removal of the tail fan. The hindgut was put in nucleic acid preservation solution (4 M ammonium sulphate, 25 mM sodium citrate, 10 mM EDTA; pH 5.5) until further processing. The individual fore- and midgut of 6 – 7 and 52 dpe lobsters (carapace length 10.8 ± 1.8 mm) was too small to reliably obtain sufficient total nucleic acids extract.

The 13 mpe lobsters were euthanised in a seawater ice slurry for at least 5 min prior to dissections. An inverted Y incision was made on the dorsal surface of the carapace prior to extracting the foregut and midgut. A cut was made on the dorsum of the abdomen to excise the hindgut. Fore-, mid- and hindgut were put in nucleic acid preservation solution until further processing.

4.3.3 Total nucleic acids extraction

Each preserved sample was homogenised in 500 μL of urea extraction buffer (4 M urea, 1 % sodium dodecyl sulfate, 0.2 M sodium chloride, 1 mM sodium citrate; pH 8.2) and 5 μL of proteinase K (Bioline Pty. Ltd., NSW, Australia), heated at 37 °C for 15 min (with 5 s of vortexing every 5 min) and then incubated on ice for 5 min. Samples were mixed with 250 μL of 7.5 M ammonium acetate (Sigma-Aldrich Co., MO, USA), vortexed for 40 s and centrifuged at 16,000 *g* for 5 min (18 °C). The supernatant was re-centrifuged at 16,000 *g* for 3 min (18 °C) and the final supernatant was mixed by inversion (40 times) with 800 μL of isopropanol with 0.4 % (v/v) pink co-precipitant (Bioline) and centrifuged at 16,000 *g* for 15 min. The pellet was rinsed with 500 μL of 70 % ethanol twice before resuspension in 50 μL of buffered water (0.05 % Triton X-100, 10 mM TRIS pH 7).

4.3.4 PCR and pyrosequencing

The V1 to V3 hypervariable regions of the bacterial 16S rRNA gene were amplified from gut samples. A sequencing adaptor was added to the 5' end of each primer and there was a sample-specific barcode in every reverse primer (Table 4-1). The 20 μL PCR reactions contained 10 μL of 2 × MyTaq HS mix (Bioline), 300 nM each of forward and reverse 16S rRNA gene primers and 1 μL of 1:10 diluted total nucleic acids extract. The PCR was conducted using a C1000^{TM} Thermal Cycler (Bio-Rad Laboratories Inc., USA) with a thermal cycling program as follows: initial melting for 3 min at 95 °C; 30 cycles of denaturation for 10 s at 95 °C, annealing for 30 s at 58 °C, extension for 15 s at 72 °C; and a final extension for 2 min at 72 °C. PCR products were examined by 1.5 % agarose gel electrophoresis. PCR for the no-template control (total nucleic acids extract was substituted with buffered water) was conducted for 33 cycles.

Table 4-1. Sequences of 16S rRNA gene primers (5’ → 3’).

Name	Adaptor	Key	Barcode	Linker	Target sequence
B16S-9F	CCTATCCCCTGTGTGC CTTGGCAGTC	TCAG	-	AC	GAGTTTGATCMTGGC TCAG
B16S-541R	CCATCTCATCCCTGCG TGTCTCCGAC	TCAG	(barcode)	AC	WTTACCGCGGCTGCT GG

PCR products were purified using SureClean (Bioline) according to the manufacturer’s instructions and concentrations determined using a Qubit^{TM} fluorometer (Invitrogen^{TM}, Life Technologies Australia Pty. Ltd., VIC, Australia). For each sample, 25 ng of purified PCR product was pooled. The pool was purified using SureClean and quantified using Qubit. A 100 µL suspension containing 2 ng µL^{-1} of amplicons was sent to Macrogen Inc. (Seoul, Korea) for pyrosequencing (454 GS-FLX+ Titanium, Roche, USA). The sampling size and experimental design were influenced by availability of cultured lobsters of a similar genetic background at important post larval and post puerulus developmental stages.

4.3.5 Data analyses

The reads sequence file was separated according to barcodes and trimmed from primers using Geneious 8.1.7 (Kearse et al., 2012). The sequences were deposited in the Sequence Read Archive under BioProject accession number PRJNA396648. The sequences were exported to the CloVR pipeline for 16S rRNA metagenomics via the Data Intensive Academic Grid computational cloud (DIAG, 2016). CloVR default settings were used. In brief, CloVR employed several software packages including Qiime and UCHIME for denoising (poor quality and chimeric sequences) and Mothur to cluster unique sequences and analyse richness and diversity indices (White et al., 2011). Clusters of filtered sequences with 95 % nucleotide sequence identity or operational taxonomic units (OTUs) were assigned to known taxa using the RDP Bayesian Classifier within CloVR at a confidence threshold of 0.5. Beta diversity estimators were calculated using phylogenetic distance to generate a weighted UniFrac principal coordinate analysis (PCoA) plot in Qiime as per White et al. (2011). Good's coverage was calculated based on the formula of (1 – [number of singleton reads / total number of reads]) × 100 %. Counts of OTU sequences in the single no-template control library (predominantly *Methylobacteriaceae*) were subtracted from those in the samples. Observed OTUs and alpha diversity estimations were compared using one-way ANOVA with Tukey *post hoc* analyses and independent samples t test in SPSS v20. A P value of less than 0.05 was considered significant. Statistically significant differences in OTU abundances between sampling groups were assessed by multiple pairwise comparisons using Metastats software where a false discovery rate-adjusted P value (Q) of less than 0.05 was considered significant. Taxonomic relative abundance charts were generated using Krona (Ondov et al., 2011) based on OTU counts at phylum, class, order and family level. Venn diagrams were drawn using InteractiVenn (Heberle et al., 2015) to show shared and unique OTUs between samples.

4.4 Results

4.4.1 Summary of pyrosequencing

After quality filtering and removal of chimeric sequences, a total of 87 127 reads were obtained from 15 samples (Table 4-2). Mean reads per sample was 5808 (4828 – 7178) and mean read length was 452 bp. Good's coverage ranged between 99.2 and 99.7 %.

4.4.2 Alpha diversity analyses

The number of observed OTUs ranged from 51 - 71 in the foregut, 76 - 104 in the midgut and 70 - 231 in the hindgut (Table 4-2). Chao, ACE and Jackknife richness estimators showed 62 - 104, 103 - 185 and 93 - 268 OTUs in the foregut, midgut and hindgut, respectively (Table 4-3). The observed OTUs, Chao, ACE and Jackknife indices were significantly higher in the hindgut compared to the foregut of 13 mpe lobsters ($P < 0.05$). This was consistent with the hindgut library being significantly more diverse than the foregut and midgut libraries of 13 mpe animals according to the nonparametric Shannon and Simpson indices ($P < 0.01$). However, there were no significant differences in the number of OTUs or bacterial richness and diversity (Chao, ACE, Jackknife, Shannon and Simpson indices) in the hindgut (6 - 7 dpe *vs* 52 dpe *vs* 13 mpe) of animals at different developmental stages ($P > 0.05$). Rarefaction curves are provided in Figure 4-1.

Table 4-2. Sampling depth for gut sequence libraries of juvenile *P. ornatus*.

Sample			Sampling depth								
ID	Developmental stage (post-emergence)*	Individual no.	Filtered sequences	Obs OTUs		Good's coverage (%)	Phylum	Class	Order	Family	Genus
Foregut											
Fg13m1	13 month	1	6411	61		99.6	3	5	5	5	3
Fg13m2	13 month	2	5501	51	a	99.7	3	5	6	4	2
Fg13m3	13 month	3	7178	71		99.6	4	5	5	5	2
Midgut											
Mg13m1	13 month	1	5694	76		99.5	5	5	3	4	2
Mg13m2	13 month	2	6183	104	ab	99.4	5	8	11	14	8
Mg13m3	13 month	3	5728	83		99.3	4	7	9	11	7
Hindgut											
HgJ11	6 - 7 d	1	5106	102		99.3	7	7	12	12	8
HgJ12	6 - 7 d	2	5378	101		99.5	4	6	12	12	11
HgJ13	6 - 7 d	3	5305	137		99.4	6	9	14	12	17
Hg52d1	52 d	1	6091	231		99.6	15	24	36	37	25
Hg52d2	52 d	2	4828	70		99.5	4	4	4	4	2
Hg52d3	52 d	3	5805	96		99.3	3	4	7	7	3
Hg13m1	13 month	1	5950	94		99.4	4	6	7	7	4
Hg13m2	13 month	2	5740	128	b	99.2	6	10	18	19	15
Hg13m3	13 month	3	6229	134		99.2	7	12	16	17	11

* 6 – 7 d and 52 d: batch 1; 13 month: batch 2.

Observed OTUs of 13 months post-emergence lobsters not sharing common letters were significantly different (*P* < 0.05).

Table 4-3. Richness and alpha diversity indices for gut sequence libraries of juvenile *P. ornatus*.

Sample			Richness estimators						Diversity indices			
ID	Developmental stage (post-emergence)*	Individual no.	Chao		ACE		Jackknife		np Shannon		Simpson	
Foregut												
Fg13m1	13 month	1	86		89		87		0.94		0.66	
Fg13m2	13 month	2	62	a	64	a	67	a	1.18	a	0.54	a
Fg13m3	13 month	3	98		104		101		1.02		0.62	
Midgut												
Mg13m1	13 month	1	103		147		107		1.44		0.50	
Mg13m2	13 month	2	139	ab	152	b	145	ab	1.89	a	0.34	a
Mg13m3	13 month	3	136		185		149		1.31		0.50	
Hindgut												
HgJ11	6 -7 d	1	133		187		138		2.88		0.10	
HgJ12	6 - 7 d	2	119		140		126		2.73		0.15	
HgJ13	6 - 7 d	3	156		169		171		2.99		0.12	
Hg52d1	52 d	1	235		245		257		2.98		0.16	
Hg52d2	52 d	2	93		94		94		1.62		0.42	
Hg52d3	52 d	3	186		268		224		2.44		0.14	
Hg13m1	13 month	1	127		183		132		2.38		0.15	
Hg13m2	13 month	2	165	b	223	b	174	b	2.76	b	0.12	b
Hg13m3	13 month	3	173		194		184		2.55		0.15	

* 6 – 7 d and 52 d: batch 1; 13 month: batch 2.

Indices of 13 months post-emergence lobsters not sharing common letters were significantly different ($P < 0.05$).

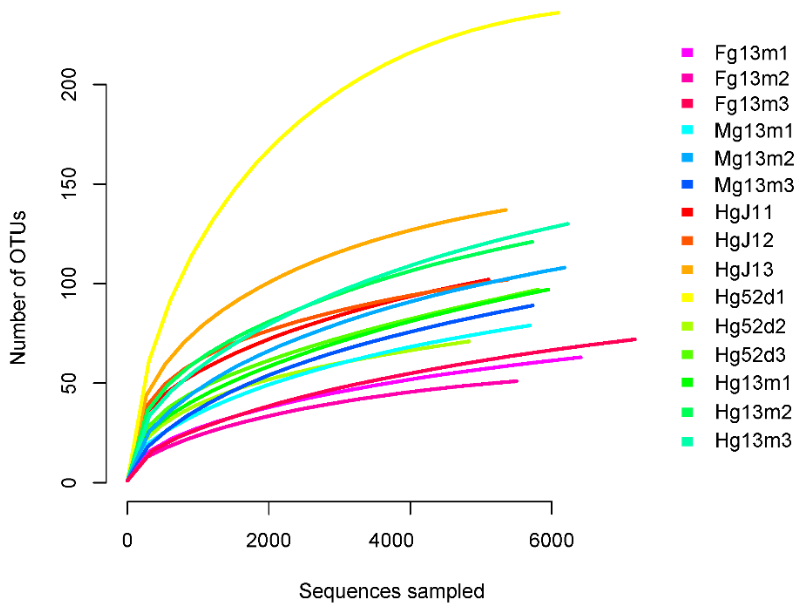


Figure 4-1. Rarefaction curves for gut sequence libraries of juvenile *P. ornatus* (see Table 4-2 for abbreviation of samples).

4.4.3 Beta diversity analyses

The weighted UniFrac PCoA plot demonstrated variation in abundance of OTUs among different samples (Figure 4-2). The first principal coordinate explained most (61 %) of the variation while the second and third principal coordinates explained 17 % and 8 %, respectively. Four core clusters were identified with the first cluster consisting of three foregut and one midgut libraries of 13 mpe animals. The second cluster was made up of two midgut libraries of 13 mpe animals and two hindgut libraries of 52 dpe juveniles. The third cluster contained three hindgut libraries of 13 mpe lobsters. Three hindgut libraries of 6 – 7 dpe juveniles formed the fourth cluster. A single hindgut library from a 52 dpe animal was dissimilar from the core clusters. The PCoA plot showed that the hindgut bacterial communities became more dissimilar from one another with increasing stage of development.

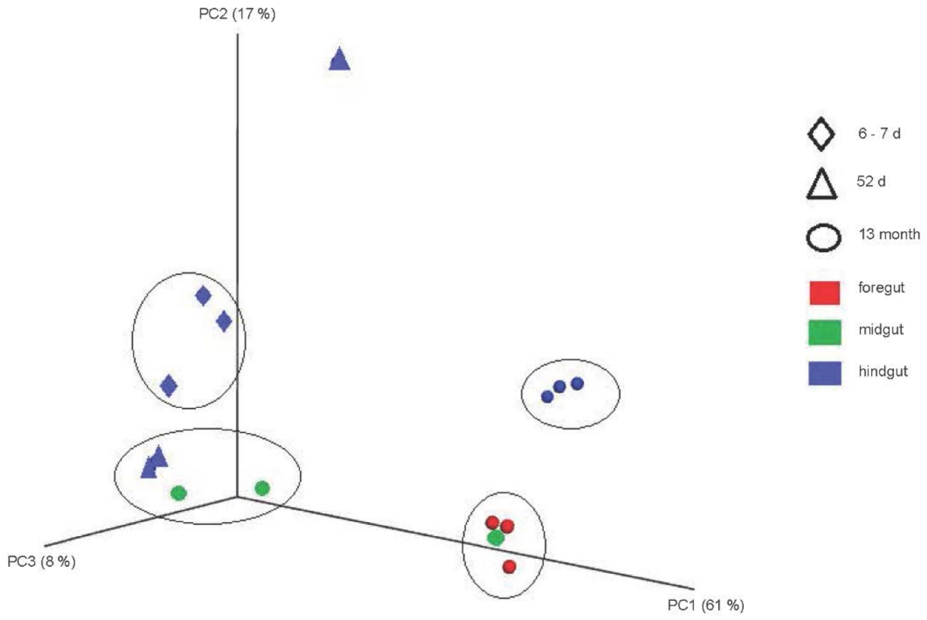


Figure 4-2. Weighted UniFrac principal coordinate analysis plot showing similarity in gut sequence libraries of juvenile *P. ornatus*.

4.4.4 Taxonomic composition

A taxonomic summary of the *P. ornatus* gut microbiomes is presented in Figure 4-3. The five main bacterial phyla represented were Tenericutes, Proteobacteria, Bacteroidetes, Actinobacteria and GN02. The three gut regions of 13 mpe lobsters were mostly represented by Tenericutes (64.2 ± 10.1 %), Proteobacteria (32.3 ± 10.1 %) and Bacteroidetes (2.6 ± 1.1 %). The hindgut of 13 mpe lobsters had a significantly higher abundance of Bacteroidetes than the foregut and midgut, and significantly more Proteobacteria than the foregut ($P < 0.05$). There were significantly fewer Proteobacteria but more Tenericutes and Actinobacteria in the hindgut compared to the midgut of 13 mpe animals ($P < 0.05$). The hindgut across different developmental stages were dominated by Proteobacteria, Tenericutes and Bacteroidetes. Tenericutes were significantly more represented ($P < 0.05$) in the hindgut of 13 mpe lobsters (65.5 ± 1.2 %) than that of 52 dpe animals (0.3 ± 0.1 %). Additionally, Bacteroidetes were significantly more abundant ($P < 0.01$) in the hindgut of 13 mpe individuals (6.8 ± 0.7 %) than that of 6 - 7 dpe juveniles (4.5 ± 2.3 %).

The top eight bacterial classes detected in the gut samples were Mollicutes, Gammaproteobacteria, Alphaproteobacteria, Saprospirae, Bacteroidia, Deltaproteobacteria, Actinobacteria and Flavobacteriia (Figure 4-3). For 13 mpe juveniles, the two major classes in the foregut were Mollicutes and Gammaproteobacteria. In the midgut and hindgut of 13 mpe lobsters, the top three classes recovered were Gammaproteobacteria, Mollicutes and Bacteroidia. Gammaproteobacteria were significantly more abundant ($P < 0.05$) in the midgut (60.1 ± 24.1 %) than the foregut (10.7 ± 3.3 %) and hindgut (24.1 ± 2.9 %) of 13 mpe animals. The five most represented classes in the hindgut across the developmental stages were Gammaproteobacteria, Alphaproteobacteria, Mollicutes, Saprospirae and Bacteroidia. Gammaproteobacteria and Alphaproteobacteria were significantly more abundant ($P < 0.05$) in the hindgut of 52 dpe animals (Gammaproteobacteria 78.6 ± 20.9 %, Alphaproteobacteria 2.4 ± 2.2 %) compared to that of 13 mpe lobsters (Gammaproteobacteria 24.1 ± 2.9 %, Alphaproteobacteria 1.8 ± 1.0 %). In contrast, Bacteroidia were significantly more represented ($P < 0.05$) in the hindgut of 13 mpe lobsters (6.7 ± 0.6 %) than that of 52 dpe juveniles (0.03 ± 0.02 %). The abundance of Mollicutes in the hindgut of 13 mpe individuals (65.5 ± 1.2 %) was significantly higher ($P < 0.01$) than that of 6 - 7 dpe (0.5 ± 0.3 %) and 52 dpe animals (0.3 ± 0.1 %).

A total of 20 major bacterial families were represented in the gut samples (Figure 4-3). For 13 mpe juveniles, the most common families in the foregut were *Pseudoalteromonadaceae* and *Mycoplasmataceae*. *Pseudoalteromonadaceae*, *Marinilabiaceae*, *Vibrionaceae* and *Mycoplasmataceae* were predominant in the midgut and hindgut of 13 mpe lobsters. *Vibrionaceae* were significantly more abundant ($P < 0.05$) in the hindgut (1.6 ± 0.3 %) than in the midgut (0.3 ± 0.1 %) and foregut (0 %) of 13 mpe lobsters, and significantly more prevalent ($P < 0.01$) in the midgut compared to the foregut. The most common bacterial families in the hindgut across different developmental stages were *Pseudoalteromonadaceae, Rhodobacteraceae, Vibrionaceae*, *Alcanivoracaceae*, *Marinilabiaceae*, *Saprospiraceae* and *Mycoplasmataceae*. A member of *Alcanivoracaceae*, genus *Alcanivorax* was significantly more abundant ($P < 0.01$) in the hindgut of 6 - 7 dpe juveniles (17.5 ± 5.5 %) compared to that of 52 dpe animals (0.02 ± 0.02 %).

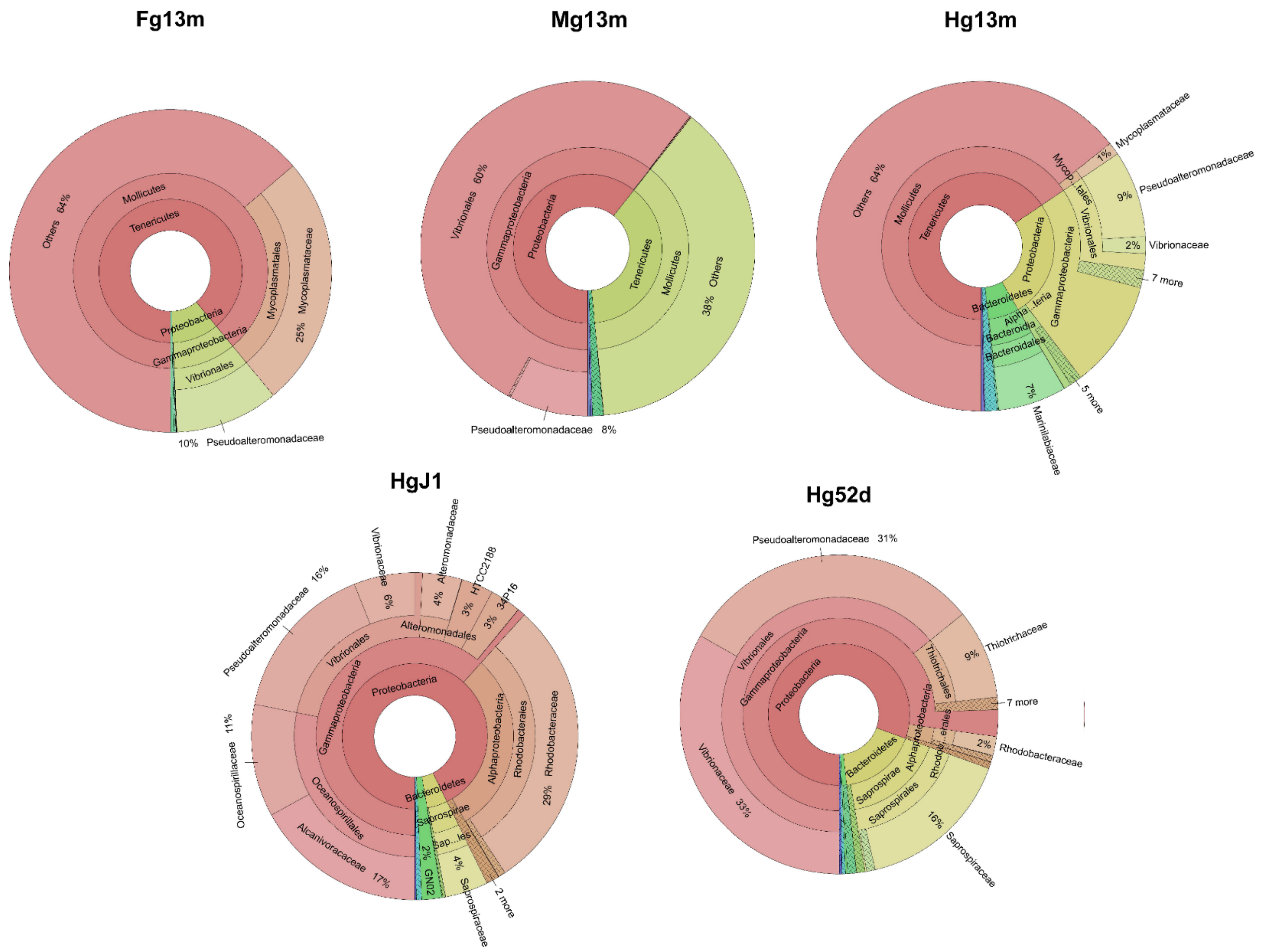


Figure 4-3. Relative abundance of OTUs in gut samples of juvenile *P. ornatus* at phylum, class, order and family levels (see Table 4-2 for abbreviation of samples).

The Venn diagrams showed that 41 OTUs (33 % of Fg13m, 24 % of Mg13m, 17 % of Hg13m) were shared by the three gut regions of 13 mpe lobsters (Figure 4-4A). For 13 mpe animals, the hindgut had the highest number of unique OTUs (141; 59 % of Hg13m) and shared more OTUs with the midgut (33 % of Hg13m) than the foregut (25 % of Hg13m). The hindgut of juveniles at different developmental stages shared 37 OTUs (18 % of HgJ1, 12 % of Hg52d, 16 % of Hg13m) (Figure 4-4B). The hindgut of 52 dpe animals had the greatest number of unique OTUs (199; 63 % of Hg52d) and shared more OTUs with 6 – 7 dpe (26 % of Hg52d) than 13 mpe lobsters (23 % of Hg52d). From the 41 and 37 shared OTUs, the core gut microbiomes were from classes Mollicutes, Gammaproteobacteria and Alphaproteobacteria.

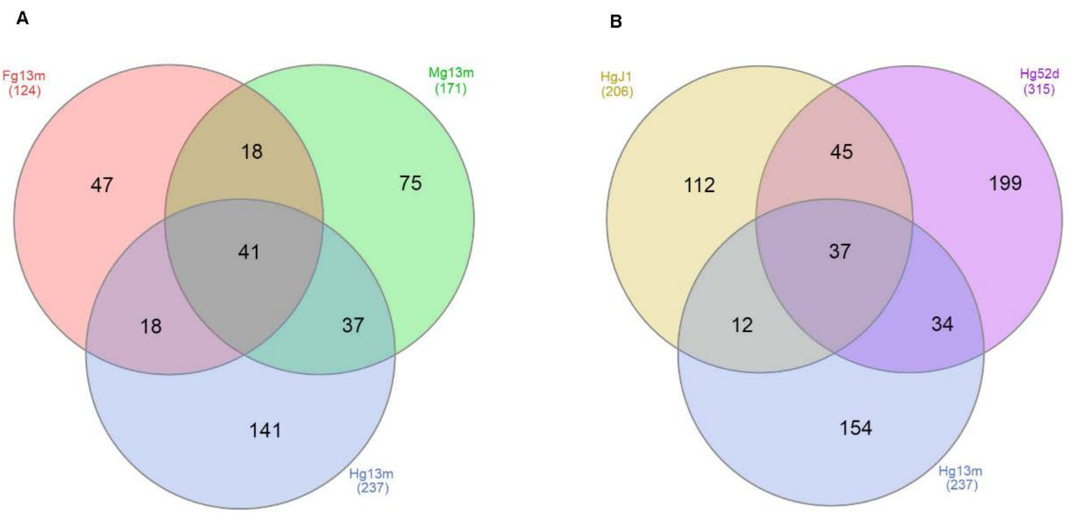


Figure 4-4. Venn diagrams showing shared and unique OTUs in (A) different gut regions and (B) different developmental stages in gut samples of juvenile *P. ornatus* (see Table 4-2 for abbreviation of samples).

4.5 Discussion

This is the first study to comprehensively explore gut microbiomes of the juvenile spiny lobster. The microbial composition was affected by gut region and the hindgut microbiome was influenced by developmental stage. For 13 mpe lobsters, alpha diversity and PCoA analyses showed that the hindgut libraries were more diverse and dissimilar when compared to the foregut and midgut libraries. This is in contrast to Cheung et al. (2015) that reported that the bacterial diversity of mid- and hindgut libraries of *N. denticulata* were not significantly different from the foregut. These differences could be attributed to Cheung et al. (2015) not analysing the mid- and hindgut separately as in our study. When the effect of developmental stage / batch was examined, the bacterial composition of the hindgut was more similar among younger juveniles (6 - 7 dpe and 52 dpe animals; batch 1) compared to more advanced juveniles (13 mpe animals; batch 2), as supported by the PCoA plot and Venn diagram. Based on diversity indices, there was no significant difference in the hindgut microbiomes between lobsters of various growth stages, which was consistent with the gut libraries of *L. vannamei* and *P. monodon* juveniles (Rungrassamee et al., 2013; Huang et al., 2014).

The core gut microbiomes of juvenile *P. ornatus* consisted of Tenericutes and Proteobacteria. Tenericutes were reported as the dominant phylum in the gut of *N. norvegicus* (Meziti et al., 2012), Chinese mitten crab *Eriocheir sinensis* (Chen et al., 2015b), mud crab *Scylla paramamosain* (Li et al., 2012) and *L. vannamei* (Zhang et al., 2014). Nevertheless, lower levels of Tenericutes were described in the digestive tract of Atlantic blue crab *C. sapidus* (Givens et al., 2013) and *P. monodon* (Chaiyapechara et al., 2012). Proteobacteria are known to be dominant in the gut microbial assemblages of a range of crustaceans including *N. norvegicus* (Meziti et al., 2010), *C. sapidus* (Givens et al., 2013), *N. denticulata* (Cheung et al., 2015), *L. vannamei* (Huang et al., 2014; Zhang et al., 2014), *P. monodon* (Rungrassamee et al., 2013), oriental river prawn *Macrobrachium nipponense* (Tzeng et al., 2015) and *M. rosenbergii* (Mente et al., 2016). A more detailed comparison of the ratio of Tenericutes to Proteobacteria among the hindguts of 6 – 7 dpe (0.01), 52 dpe (0) and 13 mpe (2.52) lobsters found that this ratio increased with age. A similar age-dependent ratio was also reported by Huang et al. (2014) who found this ratio to increase from 0 in 1-,2-,3-month-old juveniles to 0.63 in the gut of 5-month-old *L. vannamei*.

The abundance of Tenericutes in the hindgut increased from 52 dpe to 13 mpe animals. These changes could be attributed to increasing host selection due to development of the digestive system and regional influence of immune factors along the gut (Nayak, 2010; Rungrassamee et al., 2014; Bakke et al., 2015). It is also possible that the Tenericutes were enriched through niche expansion (i.e. gut surface area) and / or resisted the host immune response as the juvenile undergoes development (Rungrassamee et al., 2013). Most of the Tenericutes identified in this study were in the class Mollicutes, which included the prevalence of the genus *Candidatus* Hepatoplasma (family *Mycoplasmataceae*) in gut samples of 13 mpe lobsters. This genus was also reported in the digestive tract of *N. norvegicus* (Meziti et al., 2012) and *E. sinensis* (Chen et al., 2015b). *Candidatus* Hepatoplasma was further implicated in the survival of nutritionally stressed isopods (Fraune and Zimmer, 2008) and may suggest a potential symbiotic role in spiny lobster nutrition.

The Proteobacteria found in the gut samples were comprised mainly of members of the Gammaproteobacteria and Alphaproteobacteria. The abundance of Gammaproteobacteria was highest in the midgut, followed by the hindgut and foregut of 13 mpe lobsters. Continuous moulting throughout lobster development results in the removal and remodelling of chitinous layers in the foregut and hindgut, and this subjects the microbial communities in these regions to frequent disturbance, new colonisations and successions. Members of the Gammaproteobacteria are ubiquitous and aggressive first colonisers of marine surfaces (Dang and Lovell, 2016) and are thus likely residents of the constantly remodelled lobster fore- and hindgut regions. The more stable environment of the midgut (Rungrassamee et al., 2013) was dominated by Gammaproteobacteria, which could infer functional roles in digestion and / or habitat expansion into the midgut gland (Ceccaldi, 1989). Within Gammaproteobacteria, family *Pseudoalteromonadaceae* was detected in all three gut regions of 13 mpe lobsters and the hindgut of three developmental stages. This was consistent with previous studies on other crustaceans that found members of *Pseudoalteromonadaceae* in the gut of *S. paramamosain* (Li et al., 2012), *N. norvegicus* (Meziti et al., 2010), banana prawn *Penaeus merguiensis* (Oxley et al., 2002) and *L. vannamei* (Tzuc et al., 2014). Previous studies on *P. ornatus* larvae have shown that *Pseudoalteromonas* spp. have a beneficial relationship with the host, including producing antibacterials to reduce pathogen colonisation (Goulden et al., 2012b).

Family *Vibrionaceae* (class Gammaproteobacteria) was found in most of the gut samples. Vibrios are frequently isolated from the gut of healthy lobsters (Sugita et al., 1987; Immanuel et al., 2006; Battison et al., 2008; Meziti et al., 2012) and have putative roles in the digestion of chitin and inhibiting the colonisation of other bacteria through the production of antibiotic metabolites (Thompson et al., 2004; Wietz et al., 2010; Goulden et al., 2012b). Microbially-produced chitinases allow for the digestion of exoskeleton from food (eg. natural prey items) and the lobster exuvia following the moult (Ceccaldi, 1989; Perera and Simon, 2015). Vibrios and Pseudoalteromonads are also involved in the degradation of algal compounds (Egan et al., 2013; Martin et al., 2014) and could have a role in the digestion of algae consumed by lobsters in natural settings. Host phylogeny (regardless of whether the host is in a natural or culture environment) has an overruling selective effect on the microbiome of *P. monodon* (Rungrassamee et al., 2014) and fish (Sullam et al., 2012). Therefore, some microorganisms may be recruited to gut assemblages even if they possess no functional capacity within the host in an artificial culture environment. Interestingly, some strains of vibrios known to be opportunistic pathogens of lobsters including *V. harveyi* were isolated from animals in this study and could become problematic if the animals are immunocompromised (Shields, 2011).

Within the family *Alcanivoracaceae* (class Gammaproteobacteria), the genus *Alcanivorax* was more abundant in the hindgut of 6 - 7 dpe juveniles compared to older lobsters suggesting developmental stage-specificity. *Alcanivorax* were reported in mussel (Bayat et al., 2016) and midgut of grouper (Yang et al., 2011). Members of *Alcanivorax* can utilise oil-based hydrocarbons as a growth substrate (Schneiker et al., 2006). Interestingly, our research group has found other bacterial genera (e.g. *Oleibacter* and *Polymorphum*) capable of degrading hydrocarbons associated with the gut of *P. ornatus* puerulus (unpub. data), which is the nonfeeding lecithotrophic post larval stage that occurs before juvenile emergence. The genomes of such bacteria have shown to be enriched with proteins responsible for lipid metabolism (Nie et al., 2012), and may play a role in the lipid-dominant metabolism during puerulus development (Jeffs et al., 1999; Fitzgibbon et al., 2014). Overall, developmental stage appeared to influence the shift from Proteobacteria to Tenericutes-dominated microbiomes of the hindgut. This is presumably driven by changes in host ecophysiology. For example, emergent juveniles (i.e. 6 – 7 dpe) could harbour communities that have roles in post larval starvation resistance (Espinosa-Magaña et al., 2017) and the structure of these communities then change with the developing reptant feeding habits of juveniles (Perera and Simon, 2015).

It should be noted that some of the differences between sequence libraries across developmental stages observed in this study could be accounted for by genetic factors (Sullam et al., 2015), host selective processes (Zhang et al., 2014) and culture methods (recirculating *vs* flow-through system) (Attramadal et al., 2014). However, there is a growing body of evidence that supports host phylogeny having an overruling selective effect on the gut microbiome of crustaceans (Tzeng et al., 2015) and teleosts (Sullam et al., 2012; Bakke et al., 2015). For example, the majority of gut microbiomes of *N. norvegicus* were different from bacteria in the tank water and feed, with no apparent difference between mussel and pellet-fed lobsters (Meziti et al., 2012). Additionally, the gut microbiome of *L. vannamei* was not similar to the bacterial community of rearing seawater (Huang et al., 2014; Zhang et al., 2014). As such most of the variation in microbiomes across development may be more in line with host intrinsic factors, and not with external environmental factors.

To the best of our knowledge this is the first study showing that the core gut microbiomes of cultured *P. ornatus* juveniles are dominated by Tenericutes and Proteobacteria. The composition of the gut microbiomes was influenced by gut region and developmental stage. This was evidenced in the characterisation of the gut from 13 mpe lobsters, where the hindgut was more diverse and compositionally dissimilar to the fore- and midgut regions, and further differed in structure when compared to the hindgut of younger lobsters. Identification of commonly found gut bacteria of crustaceans including *Pseudoalteromonadaceae* and *Vibrionaceae* could infer functional significance for juvenile lobsters. With an increased understanding of the gut microbiomes of healthy lobsters we may identify specific bacterial groups as indicators of health, disease, and productivity traits and identify gut symbionts as probiotic candidates to improve lobster health.

Chapter 5 Predatory bacteria in the haemolymph of the cultured spiny lobster *Panulirus ornatus*

5.1 Abstract

Bdellovibrio and like organisms (BALOs) are obligate predators of other bacteria in a range of environments. The recent discovery of BALOs in the circulatory system of spiny lobster *P. ornatus* warrants more investigation. We used a combination of co-culture agar and broth assays and transmission electron microscopy to show a *Halobacteriovorax* sp. strain Hbv preyed upon the model prey bacterium *Vibrio* sp. strain Vib. The haemolymph microbiome and an immune parameter (percentage of granulocytes) of juvenile *P. ornatus* were characterised following injection of phosphate buffered saline (control) or prey and/or predator bacteria. The predator Hbv had no effect on survival and percentage of granulocytes compared to the control after 3 d. However, when compared to the prey only treatment group, lobsters injected with both prey and predator showed (1) significantly lower abundance of genus *Vibrio* in the haemolymph bacterial community composition; and (2) a reduction in plasma bacterial DNA concentration (bacterial load) after 2 d. This study indicates that predatory bacteria are not pathogenic and may assist in controlling microbial population growth in the haemolymph of lobsters.

5.2 Introduction

Bdellovibrio and like organisms (BALOs) are a group of Gram negative bacteria that prey on other Gram negative bacteria. This group consists of families *Bdellovibrionaceae*, *Bacteriovoracaceae*, *Halobacteriovoraceae* and *Peredibacteraceae* (Rotem et al., 2014; Koval et al., 2015). BALOs are both small and motile, conferring a physical advantage over other predatory / bacteriovorous microorganisms including bacteriophages that are smaller but nonmotile (Schade et al., 1967) and protozoans which are larger and less motile (Matz and Jürgens, 2005). BALOs exhibit either epibiotic or periplasmic modes of predation: epibiotic predators divide by binary fission while attached to prey while periplasmic predators divide by synchronous septation inside prey (Fenton et al., 2010).

BALOs act as natural top-down population control mechanisms for bacterial communities in a range of aquatic and terrestrial environments (Amat and Torrella, 1989; Davidov et al., 2006; Wen et al., 2009; Chu and Zhu, 2010) and within organs (eg. gill, shell) of aquatic animals (Kelley and Williams, 1992; Pineiro et al., 2007). There are fewer reports of BALOs within animals compared to those from the environment, however they have been detected in the gut of sturgeon (Cao et al., 2012) and snakehead fish (Cao et al., 2014b). These BALOs (*Bacteriovorax* sp. and *Bdellovibrio bacteriovorus*) were shown to protect their hosts and other aquatic animal species against infections caused by a number of aquatic bacterial pathogens (Cao et al., 2014a; Cao et al., 2014b; Cao et al., 2015). It is because of this biocontrol potential that BALOs have been suggested as an alternative treatment to antibiotics and thereby mitigating associated ecological threats of antibiotic resistance (Damron and Barbier, 2013).

Various aspects of disease management and health are currently being investigated for the ornate spiny lobster *P. ornatus* as closed life cycle aquaculture production of this species reaches the commercialisation phase under UTAS-Nexus Aquasciences Pty. Ltd. (UNA). An aspect of health was to establish baseline microbiomes of healthy lobsters. Two studies from this project have employed next generation sequencing to perform the first extensive characterisation of the haemolymph (Chapter 3) and gut (Chapter 4) microbiomes of healthy juvenile *P. ornatus*. We revealed that BALO members were present at low relative abundances in the haemolymph (*Bacteriovorax* 0.4 – 0.8 %, *Bdellovibrio* 0.3 – 4.4 %) and hindgut (*Bacteriovorax* 0.03 – 8.0 %, *Bdellovibrio* 0.1 %) of cultured juvenile *P. ornatus*. This is consistent with reported abundances of BALOs in the natural environment (e.g. water and terrestrial), which comprise less than 0.2 % of total bacteria (Rotem et al., 2014). Still, prior to this work there was no record of BALOs residing in the circulatory system of animals (Chapter 3).

BALOs are not commonly studied as they cannot be isolated by routine culture methods. Besides suitable nutrients and conditions, the isolation of BALOs require a high concentration of prey bacteria. The finding of BALOs in lobster haemolymph may infer a functional importance. The aim of this study was to screen for and characterise BALOs and examine their effect on the haemolymph microbiome and immune response of cultured *P. ornatus* juveniles.

5.3 Materials and methods

5.3.1 Prey *Vibrio* (Vib)

Vibrio sp. strain Vib was used as prey bacterium in this study. This strain was isolated from the haemolymph of a juvenile *P. ornatus* exhibiting lethargy and minimal response during handling at the Institute for Marine and Antarctic Studies (IMAS), Tasmania, Australia. The isolate was identified by Sanger sequencing and stored in 25 % (v/v) glycerol at -80 °C. Subcultures of Vib were grown in a modified marine broth consisting of 0.5 % peptone, 0.3 % yeast extract and 3.5 % Instant Ocean® sea salt (Aquarium Systems, France) at 28 °C with shaking (100 rpm) overnight. Bacteria were harvested by centrifugation at 8000 *g* for 5 min and the cell pellet was resuspended in autoclaved seawater adjusted to 35 ppt with Instant Ocean sea salt.

5.3.2 Predator *Halobacteriovorax* (Hbv)

Sea water was sampled at IMAS. The water was passed through a 3 µm filter, centrifuged at 16,000 *g* for 20 min and the concentrated suspension was further centrifuged at 1000 *g* for 5 min to separate algae from bacteria. The supernatant was cultured with harvested Vib (~10^8 cell mL^{-1}) in modified Luria broth (0.1 % Luria broth base [Sigma-Aldrich Co., MO, USA] in autoclaved sea water) at 28 °C with shaking (100 rpm). The broth co-culture was monitored daily at optical density 700 nm from opaque (0.7) until clear (0.1). The enriched co-culture was used in a double layer agar plating method, consisting of a base layer (modified Luria broth with 1.2 % agar) and overlay layer (modified Luria broth with 0.6 % agar with co-culture and ~0.5×10^{10} Vib cells). Plates were incubated at 28 °C and observed for plaques over 7 d. Individual plaques were subcultured in modified Luria broth with Vib. To avoid losses of the predator strain during processing, no attempts were made to isolate Hbv from Vib in subcultures by filtration.

5.3.3 Amplification of *Bacteriovoracaceae*-specific 16S rRNA

Broth co-cultures were initially centrifuged at low speed (1000 *g* for 5 min) to remove debris, followed by high speed centrifugation (16,100 *g* for 15 min) to concentrate bacteria. The pellet was vortexed in 200 µL of lysis buffer (7.8 M urea, 0.5 % sodium dodecyl sulphate), heated at 55 °C for 10 min and incubated on ice for 10 min. The lysate was vortexed with 100 µL of 7.5 M ammonium acetate for 30 s and centrifuged at 14,000 *g* for 5 min (4 °C). The supernatant was inverted (40 times) with 300 µL of isopropanol with 0.02 µg μL^{-1} pink co-precipitant and centrifuged at 16,000 *g* for 10 min. The pellet was washed with 500 µL of 60 % ethanol twice and resuspended in 50 µL of molecular grade water.

A semi-nested PCR was performed using universal bacterial primers and *Bacteriovoracaceae*-specific primers. Both PCR 10 µL mixtures contained 5 µL of 2 × MyTaq HS mix (Bioline Pty. Ltd., NSW, Australia), 400 nM each of 63F (5’ - CAGGCCTAACACATGCAAGTC 3’) (Marchesi et al., 1998) or Bac676F (5’ – ATTTCGCATGTAGGGGTA – 3’) (Davidov et al., 2006) for primary (63F and Bac1442R) and secondary (Bac676F and Bac14442R) PCRs respectively, and Bac1442R primer (5’ – GCCACGGCTTCAGGTAAG – 3’) and 2 µL of nucleic acids (primary PCR) or 1:10 diluted primary PCR products (secondary PCR). The PCR thermal cycling program was conducted at 95 °C for 1 min, 25 cycles (primary PCR) or 30 cycles (secondary PCR) of 95 °C for 10 s, 60 °C for 30 s and 72 °C for 15 s using CFX Connect Real-Time System (Bio-Rad Laboratories Inc., USA). PCR products were examined on a 1.5 % agarose gel. For purification, PCR amplicons were mixed with equal volumes of 19 % polyethylene glycol and 2 µL of Polyacryl Carrier (Molecular Research Center Inc., OH, USA), incubated at room temperature for 15 min and centrifuged for 16,100 *g* for 20 min. The pellet was rinsed with 70 % ethanol and centrifuged at 16,100 *g* for 15 min, twice. The pellet was resuspended in 25 µL of buffered water (0.05 % Triton X-100, 10 mM TRIS pH 7) and quantified using a Qubit fluorometer (Invitrogen, Life Technologies, VIC, Australia). Purified PCR products and *Bacteriovoracaceae*-specific primers (Bac676F and Bac1442R) were sent to Australian Genome Research Facility (AGRF, QLD, Australia) for Sanger sequencing. The results were compared with other sequences in the Ribosomal Database Project and National Center for Biotechnology Information ($\geq$ 95 % identity).

5.3.4 Transmission electron microscopy of Hbv

Multiple broth co-cultures from 1 - 6 d old were used to observe the different stages of the Hbv life cycle. The co-cultures were centrifuged at 1000 *g* for 5 min. The supernatant was deposited on Formvar/carbon grid. The sample was negatively stained with 1 % uranyl acetate and examined with a Hitachi HT7700 electron microscope at 80 kV.

5.3.5 Hbv and Vib injection in lobster

5.3.5.1 Experimental design

Panulirus ornatus were cultured from hatch at IMAS as previously described (Chapter 3). Five juveniles per treatment (67.7 ± 3.4 g) were placed in 4 × 50 L tanks within a recirculating system (temperature 28 °C; dissolved oxygen 98 %; pH 8; salinity 35 ppt). Lobsters were injected with 100 µL of either (1) phosphate buffered saline [PBS group]; (2) Vib (1 $\times 10^8$ cell mL^{-1}) [prey group]; (3) Hbv (1.04 $\times 10^8$ cell mL^{-1}) [BALO group]; or (4) Hbv and Vib (1.52 $\times 10^8$ cell mL^{-1}, combined) [prey+BALO group]. The prey and predator inoculums were harvested as described in Sections 5.3.1 and 5.3.2 respectively but resuspended in PBS. The predator bacteriolytic ability of the harvested inoculums were confirmed *in vitro* by adding an equal volume of modified Luria broth before incubation at 28 °C and colony enumeration using the drop plate technique on marine agar 2216 (Difco Laboratories Inc., MI, USA). Injection and sampling were made between the basis and the ischium of lobster pereiopods. Approximately 160 µL of haemolymph was sampled from each animal prior to injection (0 d) and additionally 1, 2 and 3 d after injection using a chilled syringe (23G needle) pre-filled with an equal volume of anticoagulant (400 mM NaCl, 0.1 M glucose, 30 mM trisodium citrate, 26 mM citric acid, 40 mM EDTA) (modified from Rodríguez-Ramos et al., 2011). All lobsters were in intermoult phase during sampling.

5.3.5.2 Bacterial analyses

Three hundred microliters of anticoagulated haemolymph from each animal was centrifuged at 500 *g* for 10 min (4 °C) and the supernatant was further centrifuged at 16,000 *g* for 10 min (4 °C) to concentrate bacteria from the plasma. All but 30 µL of the supernatant was removed and 300 µL of lysis buffer was added before storage at -20 °C.

Thawed plasma samples were added with 5 µL of proteinase K (Bioline), heated at 55 °C for 20 min (vortexed every 5 min) and put on ice for 10 min. Samples were added with 200 µL of 7.5 M ammonium acetate, vortexed for 30 s and centrifuged at 14,000 *g* for 5 min (4 °C). Six hundred microliters of isopropanol with 0.02 µg μL^{-1} pink co-precipitant was mixed with the supernatant and incubated for 15 min before centrifugation at 16,000 *g* for 30 min. The pellet was rinsed with 500 µL of 60 % ethanol twice and resuspended in 30 µL of buffered water. Each plasma extract was quantified using Qubit.

To analyse bacterial diversity, a nested PCR approach was employed due to the occurrence of nonspecific PCR products in early assays. The amplicon library was constructed from all the plasma sampled on days 1 and 3 from each treatment group. The primary PCR mixture contained 5 µL of 2 × MyTaq HS mix, 200 nM each of 27F (5’ – AGAGTTTGATCMTGGCTCAG – 3’) and 1492R (5’ – GGTTACCTTGTTACGACTT – 3’) 16S rRNA gene primers and 1 µL of plasma extract. A thermal cycling program of 95 °C for 3 min, 20 cycles of 95 °C for 15 s, 50 °C for 30 s and 72 °C for 30 s was conducted using a C1000 Thermal Cycler (Bio-Rad Laboratories Inc., USA). The secondary PCR consisting of 5 µL of 2 × MyTaq HS mix, 400 nM each of 27F and 1100R (5’ – AGGGTTGCGCTCGTTG – 3’) and 1 µL of primary PCR product was ran at 95 °C for 3 min, 28 cycles of 95 °C for 10 s, 55 °C for 30 s and 72 °C for 30 s. PCR products were purified using SureClean (Bioline) according to manufacturer’s instructions and quantified using Qubit. Twenty microliters suspension containing 2 ng μL^{-1} of PCR amplicons of each plasma and extraction control was sent to AGRF for amplicon diversity profiling 27F - 519R (Illumina MiSeq, USA).

5.3.5.3 Percentage of granulocytes

Haemolymph smears were fixed with 70 % methanol, stained with May-Grunwald and Giemsa solution and affixed with DPX mounting medium (Evans, 2003). Granulocytes consisting of small granular and large granular haemocytes (Sritunyalucksana et al., 2005) were counted from 100 haemocytes at high dry objective (400×). The percentage of granulocytes was calculated using the fraction of total granulocytes from the total haemocytes.

5.3.6 Data analyses

Paired-end amplicon reads were aligned using PEAR and trimmed from primers using Geneious 8.1.7 (Kearse et al., 2012). Chimeras were removed using UCHIME and nonchimera files were processed in CloVR pipeline (White et al., 2011) which assigned operational taxonomic units (OTUs) to known taxa based on Greengenes database using RDP Bayesian classifier with 0.8 confidence threshold. OTUs that were in the extraction controls were removed from the OTU table (CloVR output) before being uploaded to MicrobiomeAnalyst (Dhariwal et al., 2017) to examine alpha and beta diversity, core microbiome, relative abundanceand functional potential. Low abundance OTUs (≤ 2 count) with 10 % or lower prevalence in samples were removed. Two samples with low reads were excluded from analysis. Good’s coverage was calculated by [1 - (number of singleton reads / total number of reads)] × 100 %. The beta diversity was analysed by Bray Curtis, weighted and unweighted UniFrac distance based principal coordinate analysis at OTU level and PERMANOVA. Stacked bars of relative abundance of OTUs at phylum, class and family levels were generated. The functional potential of OTUs was predicted using PICRUSt (Langille et al., 2013) and presented as a functional diversity profile from the sum of abundance of each OTU for each KEGG metabolism normalised by category size. The functional association analysis was used to compare the KEGG pathways across treatment groups. The differential abundance of OTUs was compared among treatment groups using DAME (Piccolo et al., 2017). A Venn diagram with unique and shared OTUs of each treatment group was drawn using InteractiVenn (Heberle et al., 2015).

After testing for normal distribution, one-way ANOVA in SPSS v20 was used to compare plasma DNA concentration and percentage of granulocytes among sampling days in each treatment group. For all statistical analyses, a *P* value of ≤ 0.05 was considered significant.

5.4 Results

5.4.1 Cultivation and identification of Hbv

Clear plaques appeared on double layer agar plates containing Hbv and Vib, whereas control plates with only Vib exhibited an opaque lawn of confluent bacterial growth (Figure 5-1A). Plaque diameters increased with incubation duration, ranging from 1.5 mm after 5 d to 6 mm at 7 d. Broth cultures of individual plaques cleared within approximately 3 d compared to control broth that remained cloudy (Figure 5-1B). The cleared broth was identified as *Halobacteriovorax marinus* (approximately 766 bp) with ≥ 95 % identity using Sanger sequencing.

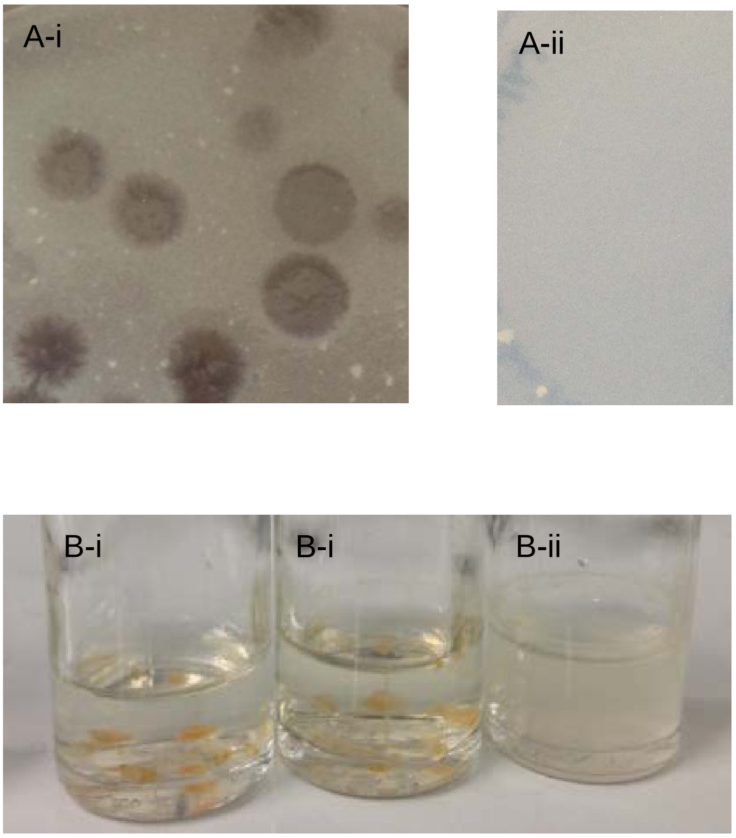


Figure 5-1. Cultivation of *Halobacteriovorax* Hbv. (A) Double layer agar plate of [i] Hbv and *Vibrio* Vib and [ii] Vib only. (B) Broth of [i] Hbv and Vib and [ii] Vib only.

5.4.2 Life cycle of Hbv

Halobacteriovorax cells were 1.55 to 2.25 µm long and 0.33 to 0.63 µm wide with a single polar flagellum. Different stages of Hbv life cycle were observed, including attachment to prey Vib (Figure 5-2A), establishment and growth within the prey periplasm (Figure 5-2B), lysis of prey which released progeny cells (Figure 5-2C), free swimming cells (Figure 5-2D) and cells with multiple pili (Figure 5-2E). The electron micrographs verified that Hbv kills Vib via the mechanism of periplasmic predation.

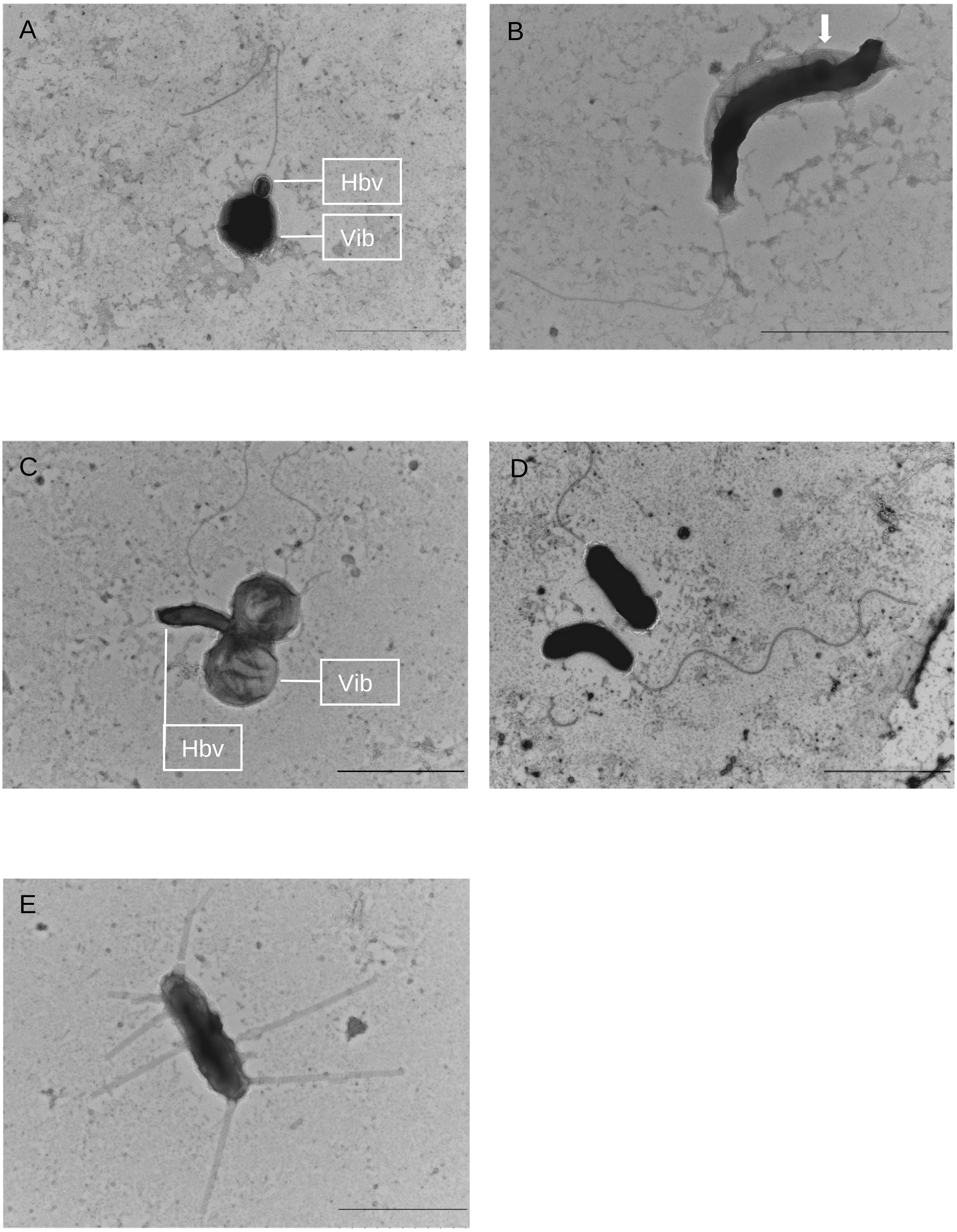


Figure 5-2. Transmission electron micrographs of life cycle of *Halobacteriovorax* Hbv. A: Attached to prey *Vibrio* Vib. B: Established inside (arrow) the prey periplasm. C: Progeny released from prey. D: Free swimming cells with flagella. E: Cell with multiple pili. All scale bars: 2 µm.

5.4.3 Hbv and Vib injection in lobster

5.4.3.1 *In vitro* bacterial culture

In vitro broth co-cultures were used to confirm that Hbv retained the ability to lyse Vib following inoculum preparation. The number of Vib colonies on marine agar decreased approximately two orders of magnitude within 2 d (Figure 5-3).

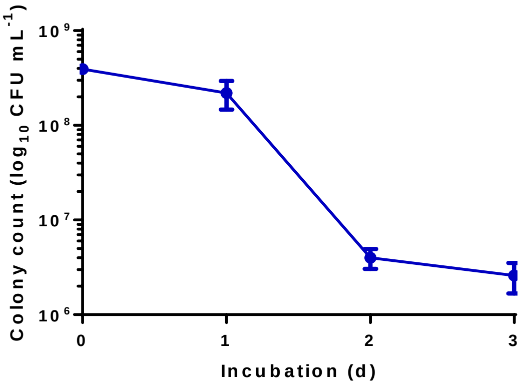


Figure 5-3. Enumeration of *Vibrio* Vib on marine agar (CFU mL^{-1}) following co-culture of *Halobacteriovorax* Hbv and Vib in modified Luria broth for 3 d. Each dot represents mean ± SEM, n = 5.

5.4.3.2 Bacterial diversity

Forty haemolymph sequence libraries yielded a total of 657,757 filtered reads with a mean of 16,443 reads per sample. Observed OTUs ranged from 13 to 146 and Good's coverage ranged from 93.1 to 99.9 % (Table 5-1). There were no significant differences (P > 0.05) in the observed OTUs, richness estimators (Chao1, ACE) and diversity indices (Shannon, Simpson) when lobster treatment groups were compared (Table 5-1).

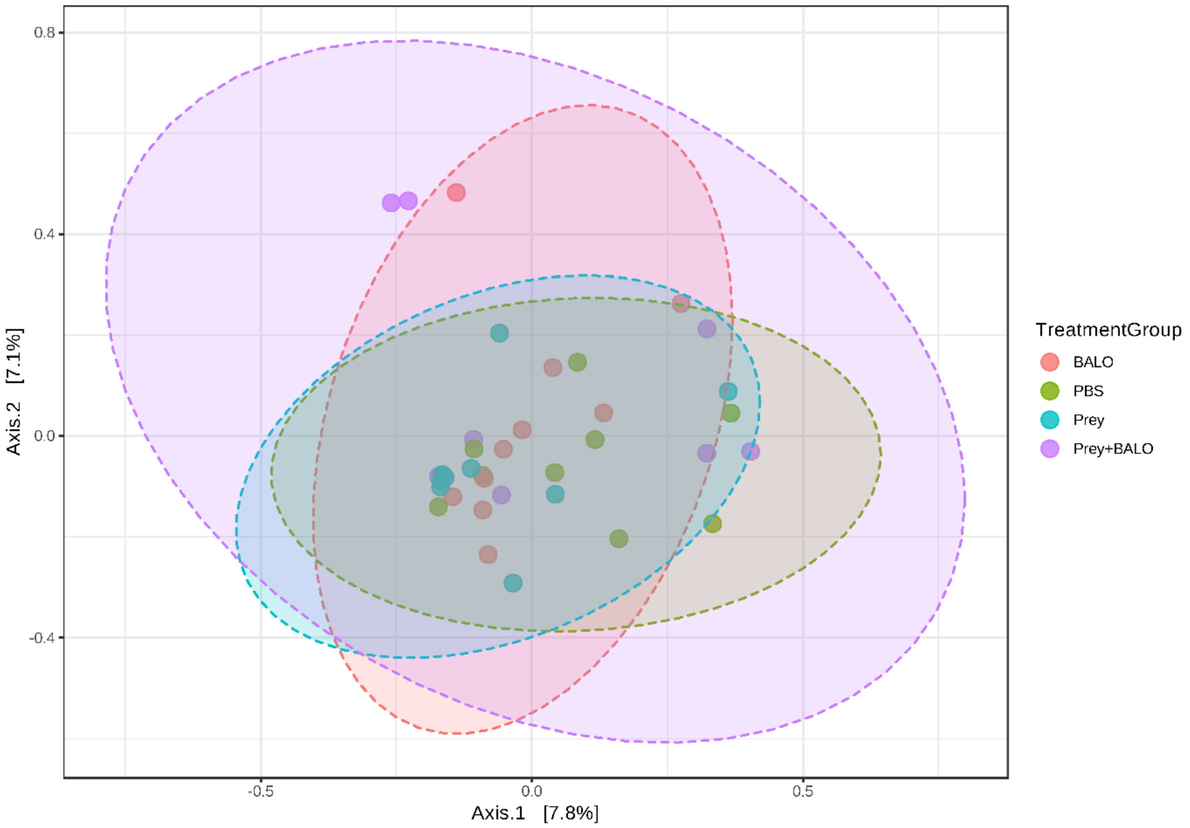
Table 5-1. Sampling depth, richness and alpha diversity indices for haemolymph sequence libraries of juvenile *P. ornatus*.

Treatment group	Lobster No.	Sampling day	Sampling depth: Filtered sequences	Obs. OTUs	Good's coverage (%)	Phylum	Class	Order	Family	Genus	Richness estimators: Chao1	ACE	Diversity indices: Shannon	Simpson
PBS	1	1	35026	106	99.5	4	8	13	17	20	114	116	2.79	0.89
		3	142	(excluded)										
	2	1	23813	113	98.7	4	6	13	16	18	129	131	2.33	0.84
		3	28426	76	98.7	4	6	11	13	10	81	86	1.32	0.55
	3	1	26650	45	99.6	4	9	14	17	13	56	53	1.85	0.76
		3	62964	99	99.3	3	6	10	13	11	108	105	1.88	0.77
	4	1	33287	28	99.8	4	7	9	12	12	39	44	0.69	0.33
		3	3178	48	93.1	3	4	7	8	6	53	56	2.49	0.87
	5	1	10630	45	98.7	3	4	6	9	6	79	70	0.36	0.12
		3	34899	13	99.9	4	6	9	10	8	41	32	0.01	0.003
prey	1	1	28536	42	99.7	4	7	15	19	16	61	60	0.37	0.15
		3	22030	19	99.8	4	7	13	13	8	28	26	0.03	0.01
	2	1	6808	69	95.8	4	7	14	18	15	78	81	1.56	0.53
		3	23596	17	99.9	3	4	7	10	9	32	43	0.02	0.003
	3	1	36698	23	99.9	3	6	12	16	11	34	40	0.01	0.002
		3	7476	14	99.6	4	6	11	13	8	15	16	2.48	0.90
	4	1	26351	60	99.7	4	3	12	16	16	65	68	1.77	0.74
		3	34959	5	99.9	2	5	4	4	4	5	5	0.02	0.01
	5	1	40342	41	99.9	3	5	10	15	14	52	54	1.45	0.71
		3	63435	18	99.9	3	4	10	11	10	25	30	0.01	0.001

Treatment group	Lobster No.	Sampling day	Sampling depth								Richness estimators		Diversity indices	
			Filtered sequences	Obs. OTUs	Good's coverage	Phylum	Class	Order	Family	Genus	Chao1	ACE	Shannon	Simpson
BALO	1	1	44010	86	99.7	4	7	16	21	19	114	111	0.80	0.27
		3	39747	20	99.9	4	6	12	14	10	23	25	0.01	0.002
	2	1	34226	62	99.5	4	8	12	14	15	76	79	0.96	0.49
		3	44736	50	99.9	4	8	11	14	14	53	54	1.51	0.70
	3	1	27240	108	99.5	4	7	14	18	18	128	131	2.37	0.86
		3	36925	125	99.2	4	5	10	14	19	140	150	2.70	0.87
	4	1	17722	24	99.9	4	6	9	13	13	30	36	1.01	0.59
		3	45975	16	99.9	4	7	11	12	9	17	19	0.50	0.30
	5	1	24526	98	99.6	3	5	8	13	18	109	112	2.32	0.82
		3	22117	14	99.9	5	7	10	11	10	20	29	2.38	0.88
prey +	1	1	52343	146	98.7	4	6	13	18	23	150	155	2.85	0.90
BALO		3	47464	44	99.8	5	6	8	10	10	62	51	1.06	0.52
	2	1	37763	102	99.1	5	7	11	16	16	117	118	2.12	0.81
		3	62597	42	99.9	4	7	12	12	11	44	46	1.26	0.67
	3	1	15848	26	99.7	5	6	7	11	12	32	45	0.54	0.25
		3	244	(excluded)										
	4	1	58564	39	99.9	5	6	12	15	15	54	60	0.31	0.10
		3	40699	21	99.9	4	5	10	13	14	51	56	0.63	0.43
	5	1	48254	24	99.9	4	7	13	17	16	30	33	0.83	0.53
		3	25901	65	99.0	5	8	13	18	17	84	88	1.17	0.53

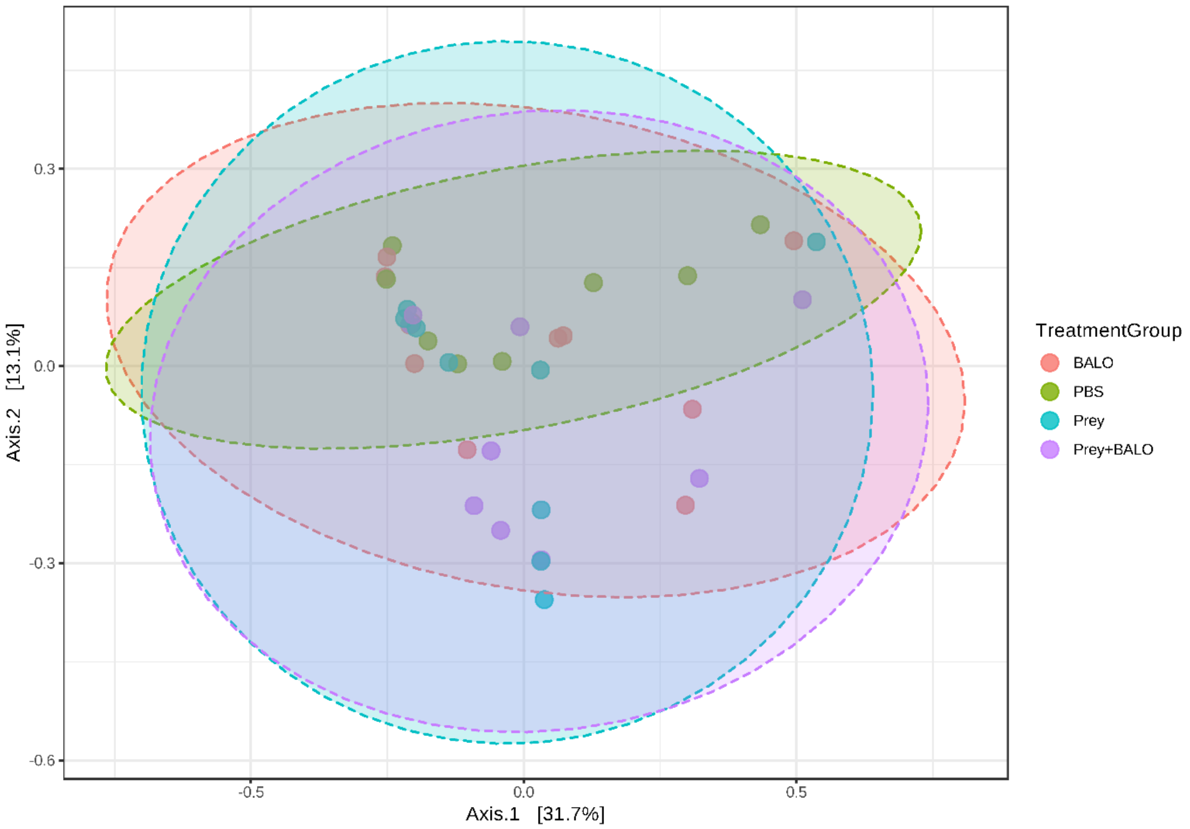
The two axes of principal coordinate analysis based on Bray Curtis, weighted UniFrac and unweighted UniFrac plot explained 14.9, 44.8 and 37.8 % of the variations in abundance of OTUs among different samples (Figure 5-4). This variation was not related to treatment groups except for unweighted UniFrac where there was some separation along the first axis. The PCoA results were supported by PERMANOVA. When the Bray Curtis index (P = 0.183), weighted UniFrac (P = 0.180) and unweighted UniFrac (P = 0.002) distance matrices were analysed statistically using PERMANOVA, only the last showed significant difference among the four types of treatment groups.

(A)



0.8
0.4
0.0
-0.4
Axis.2 [7.1%]
-0.5
0.0
0.5
Axis.1 [7.8%]
TreatmentGroup
BALO
PBS
Prey
Prey+BALO

(B)



0.3
0.0
-0.3
-0.6
Axis.2 [13.1%]
-0.5
0.0
0.5
Axis.1 [31.7%]
TreatmentGroup
BALO
PBS
Prey
Prey+BALO

(C)

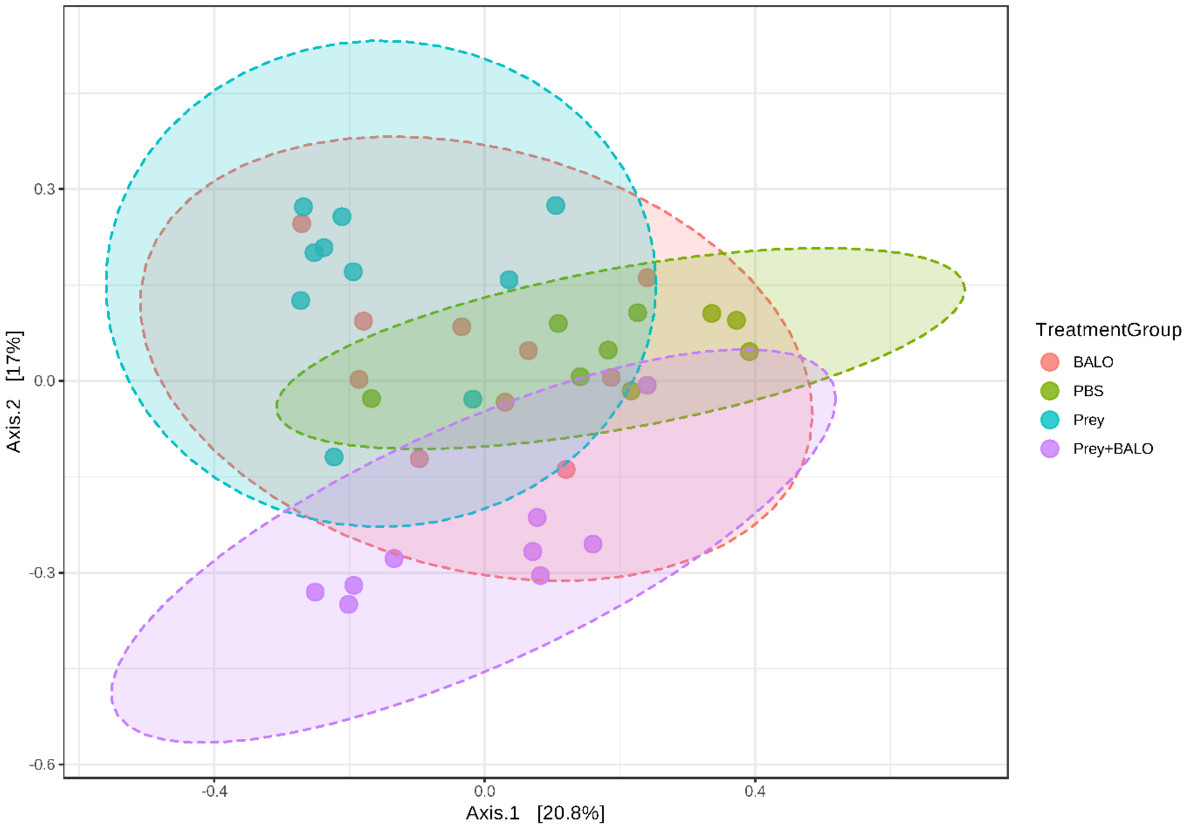


Figure 5-4. (A) Bray Curtis, (B) weighted UniFrac and (C) unweighted UniFrac principal coordinate analysis plots showing similarity in haemolymph sequence libraries of *P. ornatus* juveniles.

Sequencing of all the haemolymph libraries based on OTUs grouped by phylum indicated that the core microbiome consisted of Proteobacteria and Bacteroidetes (Figure 5-5). The Venn diagram showed that the four treatment groups shared 204 OTUs (2 % of total OTUs) belonging to phyla Proteobacteria, Bacteroidetes, Firmicutes and Actinobacteria (Figure 5-6). The haemolymph of PBS group shared the highest number of OTUs (i.e. 1194; 15 % of total OTUs) with that of prey+BALO group, while the haemolymph of prey group shared the least OTUs (i.e. 440; 5 % of total OTUs) with that of BALO group. The haemolymph libraries of prey group had the highest percentage of unique OTUs (1223; 61 % of prey group).

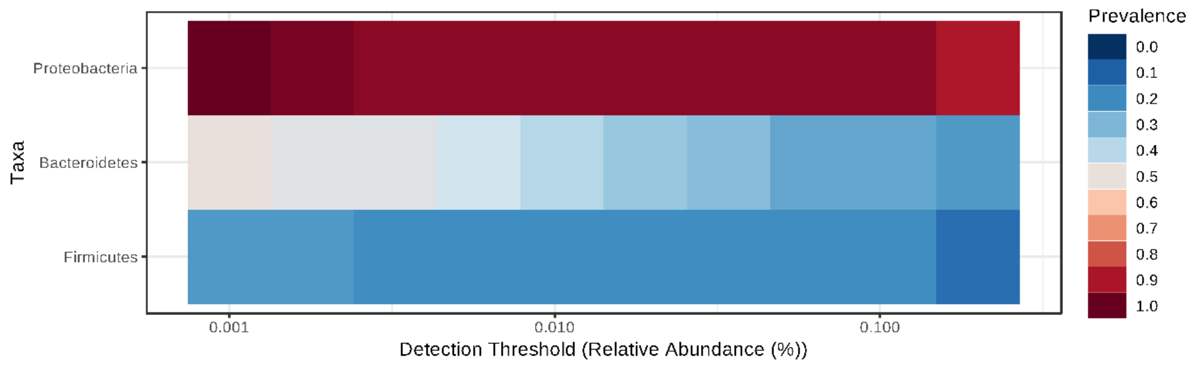


Figure 5-5. Core microbiome analysis based on relative abundance and prevalence of bacterial phyla in haemolymph libraries of *P. ornatus* juveniles.

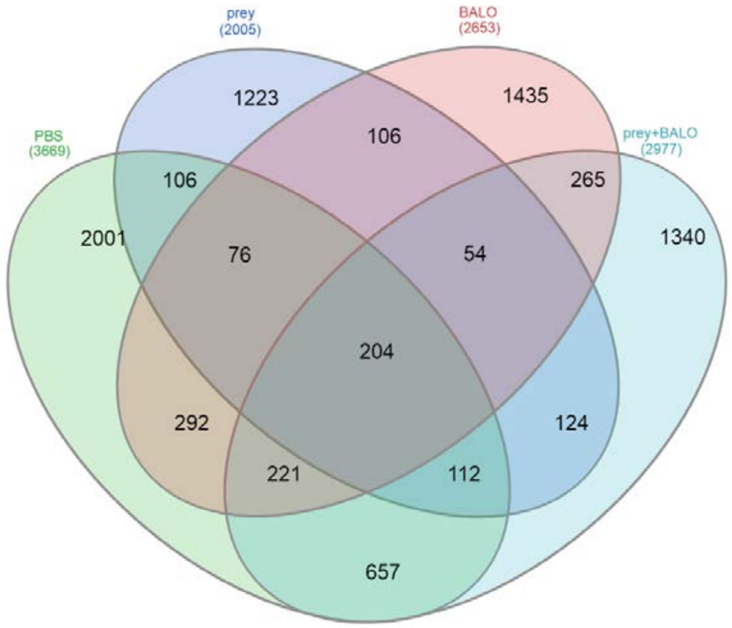
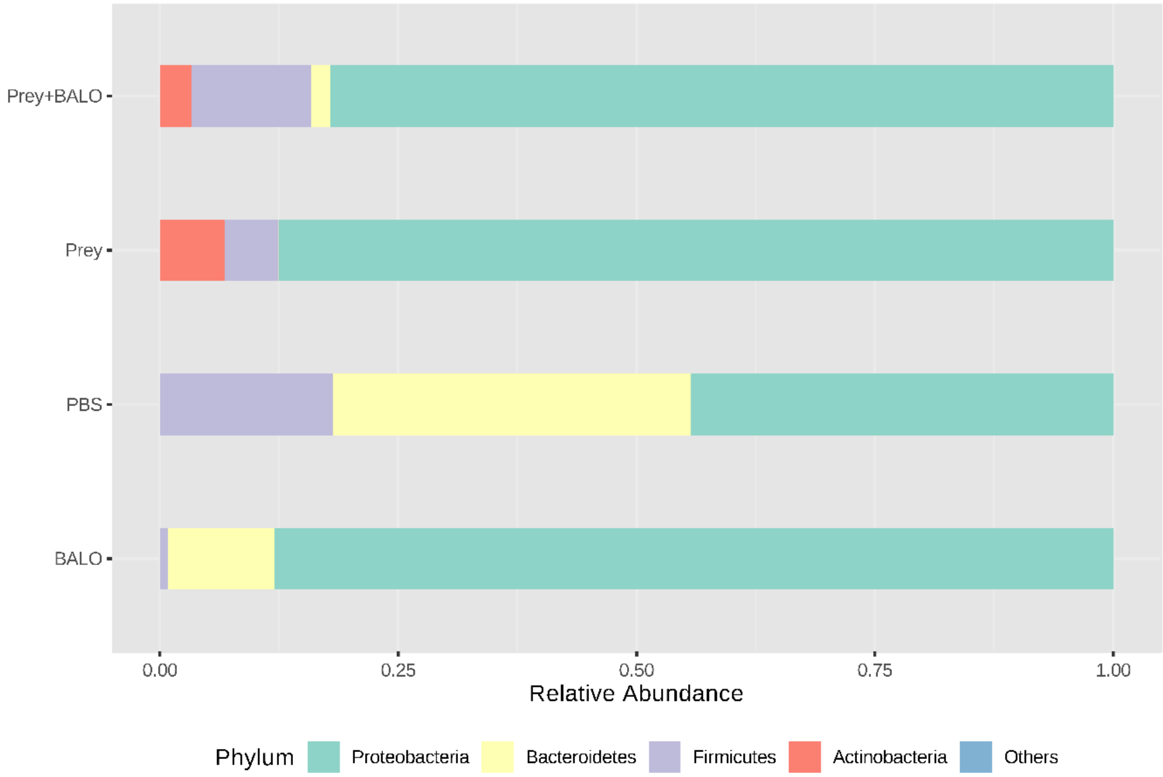


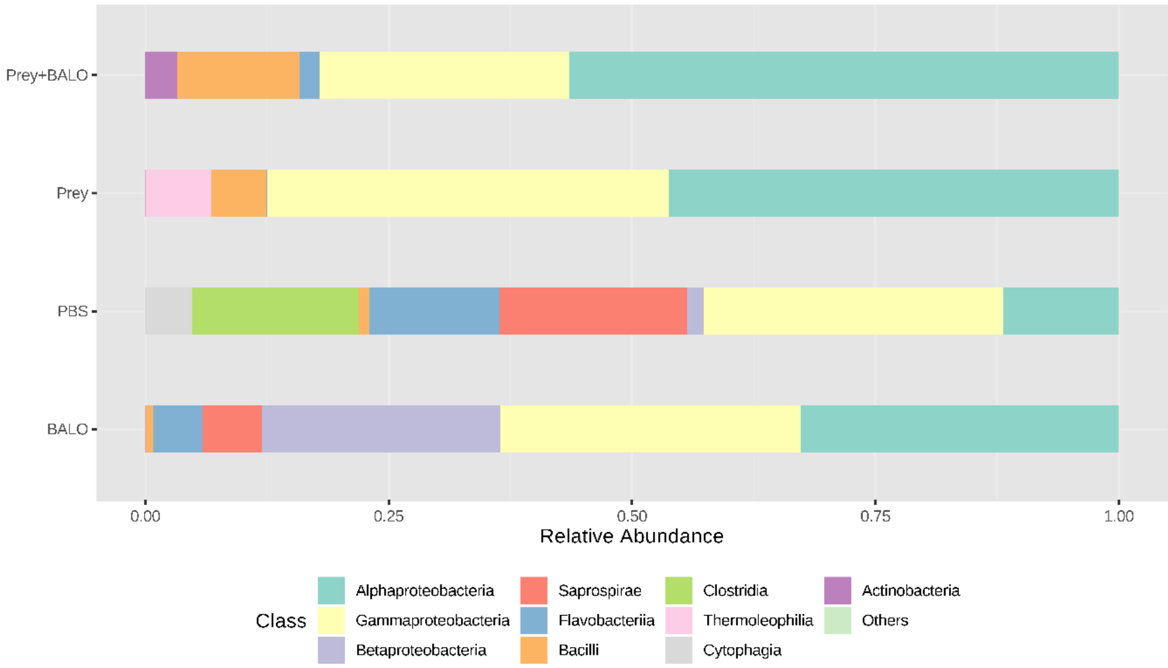
Figure 5-6. Venn diagram showing shared and unique OTUs in haemolymph libraries of *P. ornatus* juveniles.

The top three phyla in the haemolymph of lobsters were Proteobacteria (76.5 ± 5.0 %), Bacteroidetes (11.1 ± 3.3 %) and Firmicutes (8.6 ± 3.5 %) (Figure 5-7). The three most represented classes in the haemolymph of juveniles were Gammaproteobacteria (39.1 ± 6.0 %), Alphaproteobacteria (32.4 ± 5.8 %) and Bacilli (5.5 ± 2.4 %). *Rhodobacteraceae* (18.4 ± 4.6 %), *Pseudoalteromonadaceae* (11.2 ± 3.2 %), *Enterobacteriaceae* (4.7 ± 2.9 %) and *Flavobacteriaceae* (4.6 ± 2.0 %) were predominant families in the haemolymph libraries. Family *Rhizobiaceae* (11.4 ± 11.1 %; $P = 0.006$) was significantly more abundant in the haemolymph of prey+BALO lobsters than the other treatment groups. The haemolymph of prey lobsters had significantly more of genus *Vibrio* (13.7 ± 10.0 %; $P = 0.003$) but less of genus *Pseudoalteromonas* (9.2 ± 9.0 %; $P = 0.015$) represented than other treatment groups. The haemolymph of BALO animals had significantly higher abundance of genus *Tenacibaculum* (4.1 ± 2.2 %; $P = 0.003$) than the prey+BALO and prey groups. Additionally, the genus *Octadecabacter* (10.1 ± 5.4 %; $P < 0.001$) was significantly more abundant in the haemolymph libraries of BALO lobsters compared to the prey and PBS groups. However, the abundance of injected Hbv was not significantly different among treatment groups.

(A)


Prey+BALO
Prey
PBS
BALO
0.00
0.25
0.50
0.75
1.00
Relative Abundance
Phylum
Proteobacteria
Bacteroidetes
Firmicutes
Actinobacteria
Others

(B)


Prey+BALO
Prey
PBS
BALO
0.00
0.25
0.50
0.75
1.00
Relative Abundance
Class
Alphaproteobacteria
Gammaproteobacteria
Betaproteobacteria
Saprospirae
Flavobacteriia
Bacilli
Clostridia
Thermoleophilia
Cytophagia
Actinobacteria
Others

(C)

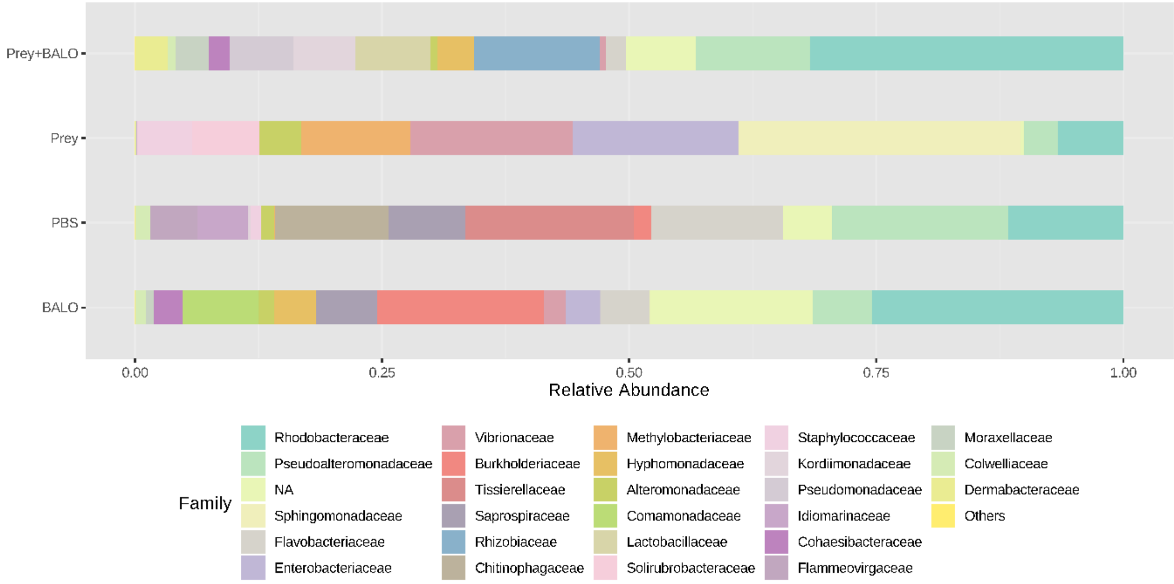


Figure 5-7. Relative abundance of OTUs in haemolymph libraries of juvenile *P. ornatus* at (A) phylum, (B) class and (C) family levels.

PICRUSt was used to predict gene families found in lobster haemolymph communities and metabolic functional profiles were consequently applied using KEGG (Figure 5-8). The four most predicted functions were metabolism of other amino acids, amino acid metabolism, metabolism of cofactors and vitamins and carbohydrate metabolism. There was no significant difference ($P > 0.05$) in potential functions across treatments.

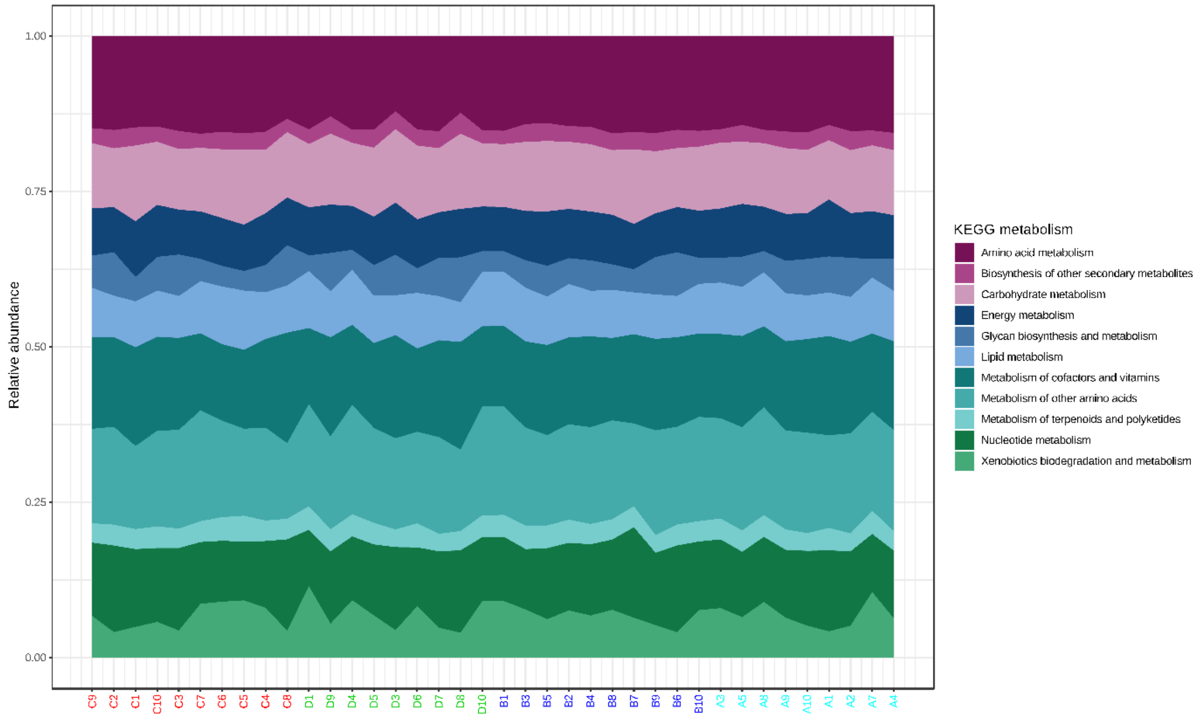


Figure 5-8. Functional diversity profiling of OTUs in haemolymph samples of juvenile *P. ornatus* based on KEGG metabolism using PICRUSt. Key: D: PBS; B: prey; C: BALO; A: prey+BALO.

5.4.3.3 Plasma DNA concentration

Plasma DNA concentration was used to estimate the amount of bacteria present in the haemolymph. Of all the treatment groups, only prey+BALO group significantly differed ($F = 10.92$, $df = 3, 16$, $P < 0.001$), where plasma DNA concentrations decreased over day 2 – 3 compared to day 0 – 1 (Figure 5-9).

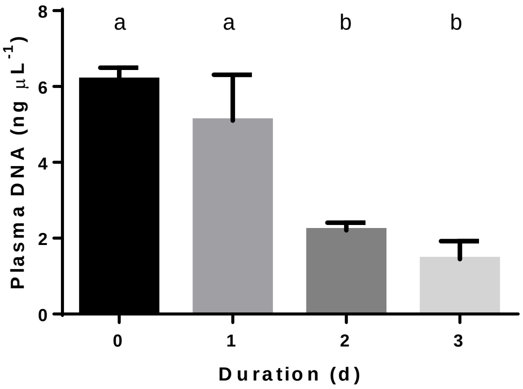


Figure 5-9. Plasma DNA concentration (ng µL^{-1}) of *P. ornatus* juveniles injected with prey+BALO. Each bar represents mean + SEM, n = 5. Means not sharing common letters were significantly different (P < 0.001).

5.4.3.4 Percentage of granulocytes

Percentage of granulocytes did not differ significantly within treatments across the sampling period (P > 0.05). Total granulocytes ranged between 3 and 66 % (mean 30 %).

5.5 Discussion

The present study is the first to report on the inoculation and effect of a BALO in the circulatory system of crustacean species. In our short term experiments, Hbv injected singularly at approximately 10^7 cells per animal had no apparent pathogenic effect towards *P. ornatus* juveniles following observations that survival and bacterial community diversity or predicted function did not significantly change when compared to control (PBS injected) animals. This discovery is consistent with other studies of BALO injection in rats (Shatzkes et al., 2017), zebrafish (Willis et al., 2016) and New York worms (Shanks et al., 2013) which did not incur pathogenic effects.

We used a combination of techniques including *in vitro* co-culture (broth and agar) and transmission electron microscopy to demonstrate Hbv preyed upon the model prey Vib. The finding of Hbv as a periplasmic predator is consistent with a report of another marine BALO, *Bacteriovorax* sp. DA5 (Wen et al., 2014). One of the benefits of periplasmic predation is that internal resources of prey cells can be accessed which reduces the dependence on obtaining nutrients from the environment, where supplies may be variable or depleted. A further advantage of BALOs within prey cells is that they have less exposure to host's immune defences such as opsonins and prophenoloxidase pathway which are induced by lipopolysaccharides (Vazquez et al., 2009). In the present study, the attack phase of Hbv involved using its nonflagellated pole to attach and invade Vib, forming a bdelloplast. From here, Hbv appeared to undergo filamentous growth, septation into progeny cells, and exit of the exhausted prey through pores as reported by Fenton et al. (2010). The single polar flagellum of the mature free swimming attack phase Hbv was almost certainly used for directional propulsion via chemotaxis upon detecting high concentrations of Vib prey (Rotem et al., 2014). Interestingly, the Hbv isolate also harboured multiple pili. Type IV pili have been reported on the nonflagellated pole of *Bdellovibrio* with functions in prey attachment, penetration and replication (Evans et al., 2007; Mahmoud and Koval, 2010). Unlike those reports, the isolate in the present study had 7 to 8 pili randomly distributed on the cell surface. Nevertheless, a variety of morphological forms of BALO isolates can exist due to the complexity of host-dependent and -independent phase of their life cycles (Richards et al., 2012).

The core haemolymph microbiome of *P. ornatus* juveniles comprised Proteobacteria and Bacteroidetes, with predicted functions primarily associated with the metabolism of amino acids, cofactors and vitamins, and carbohydrates. This is in agreement with an earlier study of older *P. ornatus* (162.7 ± 4.6 g wet weight) juveniles from the same facility (Chapter 3). Taken together, these studies suggest a degree of haemolymph microbiome stability throughout development that contribute positive functional roles to host physiology, nutrition and health. The four most common bacterial families found across all treatment groups were *Rhodobacteraceae* (e.g. *Octadecabacter*, *Phaeobacter*, *Tropicibacter*, *Loktanella*, *Ruegeria*, *Nautella*), *Pseudoalteromonadaceae* (e.g. *Pseudoalteromonas*), *Enterobacteriaceae*and *Flavobacteriaceae* (e.g. *Tenacibaculum*). A high prevalence of *Rhodobacteraceae* and *Flavobacteriaceae* was demonstrated also in an earlier study of older *P. ornatus* juveniles (Chapter 3). Members of *Rhodobacteraceae* such as *Phaeobacter* spp. and *Ruegeria* spp. can produce antibiotics that inhibit the colonisation and growth of pathogenic bacteria (Pujalte et al., 2014). Antibiotics are also produced *by Pseudoalteromonas* spp. (Wietz et al., 2010), which have been recovered from wild-captured and cultured *P. ornatus* larvae (Goulden et al., 2012b). *Loktanella* spp. have been isolated from nudibranch cerata (Doepke et al., 2012) and sea anemone (Du et al., 2010). Although *Flavobacteriaceae* are recognised for their ability to decompose organic compounds (Bernardet and Nakagawa, 2006), one of its genera *Tenacibaculum* spp. have been associated with diseased fish (Habib et al., 2014).

Following inoculation of juvenile *P. ornatus* with prey and/or predator, we found very limited detection of *Halobacteriovorax* in amplicon diversity profiling. This could be attributed to amplification biases of the three pairs of PCR primers or possible removal of the bacteria by host immune response before 24 h. Furthermore, the taxonomic assignment of OTUs could have been confounded by older classification systems present in genomic databases. For example, marine *Bacteriovorax* (*Bacteriovoracaceae*) have recently been reclassified as *Halobacteriovorax* (*Halobacteriovoracaceae*) (Koval et al., 2015), inferring that *Bacteriovorax* sequences found in the haemolymph in the present study likely includes marine strains. It is possible also that sequenced *Bacteriovorax* and *Vibrio* found in our microbiomic analyses are part of resident microbiota, given both genera were identified in earlier studies of *P. ornatus* (Chapter 3).

Nonetheless, there were several measured haemolymph parameters of prey+BALO injected juveniles that were significantly different when compared to other treatment groups. This included exhibiting a significantly lower abundance of *Vibrio* compared to the prey only treatment group, which could indicate that the injected Vib was predated by Hbv. Moreover, we found prey+BALO injected animals had significantly reduced quantities of plasma bacterial DNA after 2 d, inferring an effect on haemolymph bacterial load or that free swimming BALOs stimulated an immune response. This may be due to a top-down regulation in the haemolymph by predatory Hbv, which we demonstrated also caused a 100 fold decrease in Vib after 2 d in *in vitro* cultures. Taken together, this indicates that BALOs may assist lobsters in controlling bacterial population numbers in the haemolymph. In humans, a lower abundance of intestinal BALOs (eg. *Bdellovibrio bacteriovorus*) were found to be associated with intestinal diseases when compared to healthy individuals (Iebba et al., 2013). Indeed, the attraction of BALOs as an alternative to antibiotic treatment lies within their broad prey spectrum activity mediated through attachment to the ubiquitous cell surface lipopolysaccharides of Gram negative bacteria. This makes it exceedingly difficult for a range of bacterial hosts to develop resistance (Rotem et al., 2014).

As our haemolymph microbiome analyses could indicate an effect on Vib populations by Hbv, the use of BALOs as biocontrol agents of specific lobster pathogens warrants further study. A number of animal models (eg. worms and zebrafish) using BALOs to control human pathogens have shown promising results (Shanks et al., 2013; Willis et al., 2016). Moreover, BALOs have been administered to culture water to successfully treat pathogens of Pacific white shrimp *L. vannamei* (Cao et al., 2014a; Wen et al., 2014; Cao et al., 2015; Kongrueng et al., 2017), black tiger shrimp *P. monodon* (Li et al., 2014), goldfish *Carassius auratus* (Chu and Zhu, 2010), snakehead fish *Ophiocephalus argus* (Cao et al., 2014b), Eastern oyster *Crassostrea virginica* (Richards et al., 2012) and through feed in Chinese white shrimp *Fenneropenaeus chinensis* (Xu et al., 2007).

This is the first study to demonstrate that the BALO *Halobacteriovorax* sp. Hbv had no negative effect on *P. ornatus* upon injection into the haemolymph. Animals injected with both predator Hbv and prey *Vibrio* sp. Vib showed significant differences in haemolymph bacterial composition and load compared to other treatment groups. Recommendations for future *in vivo* studies include successfully treating definitive pathogens of juvenile lobsters with BALOs, exploration of different methods of BALO administration, and observation of long term effects of BALO administration over a range of lobster developmental stages. Such studies will be required to explore the use of BALOs as a treatment option for systemic bacterial diseases in lobsters.

Chapter 6 General discussion

6.1 Haemolymph microbiome

The presence of bacteria in the haemolymph of all the sampled healthy cultured spiny lobsters (Chapters 3 and 5) shows that asymptomatic bacteraemia is not a transient condition and is more likely to be associated with autochthonous resident bacteria. This premise is further strengthened by the high diversity of bacteria in the circulatory system, which was found to consist of up to 24 families. It was demonstrated that the haemolymph microbiome of *P. ornatus* is dominated by phyla Proteobacteria and Bacteroidetes.

The presence of resident bacteria in the open circulatory system could infer an evolutionary arms race between host and symbiont where the lobster immune system is the major factor in forging host-bacteria coevolution (Desriac et al., 2014). The lobster immune system may control the proliferation and diversity of bacteria using antimicrobial peptides, pattern recognition receptors, and prophenoloxidase enzymes (Wang and Wang, 2015). A number of studies on shrimps (*M. japonicus* and *P. monodon*) have shown that by silencing the expression of these immune factors using RNA interference, haemolymph bacterial load and mortality increased (Fagutao et al., 2009; Kaizu et al., 2011; Ponprateep et al., 2012; Wang et al., 2014). Possible adaptations by the haemolymph microbiome to resist the host immune system include changing cell envelopes to avoid recognition, producing proteases to degrade antimicrobial peptides, and secreting extracellular enzymes that inhibit phagocytosis (Wang and Wang, 2015).

Unlike bacteria in gut microbiomes, the functional roles of bacteria residing in circulatory systems are not well understood. The presence and persistence of bacterial diversity in healthy *P. ornatus* haemolymph described in Chapters 2, 3 and 5 is suggestive of positive roles in health. Haemolymph bacteria that were consistently recovered from healthy lobsters included genera *Ruegeria* and *Phaeobacter* of the family *Rhodobacteraceae* (Chapters 3 and 5), with *Ruegeria* also isolated from the haemolymph in Chapter 2. The reputed production of antimicrobials by *Ruegeria* strains (Brinkhoff et al., 2004; Bruhn et al., 2007) may complement lobster immunity in controlling haemolymph microbial populations. Similarly, studies of oysters have shown haemolymph bacteria assist the immune system directly by producing bacteriocins that compete with invasive pathogens (Defer et al., 2013); and indirectly by stimulating basal immunity to prepare the host against pathogens (Schmitt et al., 2012).

Predicted functions of haemolymph OTUs based on KEGG pathways (Chapters 3 and 5) revealed bacterial communities primarily exhibited amino acid metabolism, metabolism of other amino acids, and metabolism of cofactors and vitamins. Taken together, spiny lobsters could be dependent on haemolymph bacteria to produce amino acids, non-protein amino acids, vitamins and cofactors essential to health (Maynard, 1960; Takeuchi and Murakami, 2007). Similarly, most bacterially-produced antimicrobial compounds are proteins or peptides comprising basic units of amino acids (Coutinho et al., 2008). Haemolymph bacteria may provide trace and / or more bioaccessible forms of nutrients not adequately provided by formulated feeds, and this warrants further investigation.

A number of *Vibrio* strains are known to cause systemic diseases in spiny lobsters (Shields, 2011). Vibrios were sequenced in the haemolymph of experimentally injected lobsters (Chapter 5), but not found in apparently healthy control lobsters (Chapter 3). Sequencing of a haemolymph isolate from an abnormal individual lobster (Chapter 2) was identified also as a member of the vibrios (*V. rotiferianus*). This species is part of the well-known Harveyi clade which contains a number of strains pathogenic to marine organisms (Cano-Gomez et al., 2011). These observations may infer vibrios are transient, opportunistic, or possibly even pathogenic residents of *P. ornatus* haemolymph. Zha et al. (2018) reported that *Vibrio crassostreae* strains isolated from the haemolymph of *J. edwardsii* exhibiting tail fan necrosis were genetically distinct from strains isolated from animals without the disease. The apparent virulence of the strains associated with the diseased lobsters could be explained by selective effects of certain nutrients / substrates (Jaiswal et al., 2016), invasion of new ecotypes, dysbiosis, and horizontal transmission of virulence factors (Lerner et al., 2017).

As natural microbial population control agents, BALOs were found at a low relative abundance in the haemolymph of *P. ornatus* (Chapter 5). The bacteriolytic behaviour of BALOs are viewed as alternatives to antibiotics (Damron and Barbier, 2013) and successful injections into the lobster circulatory system showed promising potential (Chapter 5). It is hypothesised that the lobster haemolymph can provide a selective environment for BALOs to work with host immune systems to control the threat of bacterial overpopulation.

A summary of baseline haemolymph bacterial and immune parameters of healthy *P. ornatus* is provided in Table 6-1. It is envisioned that this information will be foundational to future health and disease studies for cultured spiny lobsters. It was found that the ratio of Proteobacteria to Bacteroidetes (2.3 – 32.4: 1) was skewed in favour of the former, and other decapods such as Atlantic blue crab *C. sapidus* (13.8) fall within the same range (Givens et al., 2013). However, the high end of the bacterial load range found using a culture-dependent method was approximately five and ten times more than that of acclimated wild *P. ornatus* (Sang and Fotedar, 2010) and *P. cygnus* (Evans et al., 2002), respectively. Held and stressed wild *C. sapidus* also had 3 times higher bacterial load than those that were freshly caught (Welsh and Sizemore, 1985). When cultured *P. ornatus* (Chapter 3) and wild-acclimated *P. cygnus* (Evans, 2003) were compared, the total haemocyte count falls within the baseline range but the percentage of granulocytes and clotting time do not. The differences may be due to several factors such as moult stage, season (nutritional status), and diurnal fluctuations associated with haematopoiesis (Söderhäll, 2016).

Table 6-1. Baseline haemolymph bacterial and immune parameters of healthy *P. ornatus*.

Parameter	Range	Reference
Core microbiome (Proteobacteria: Bacteroidetes)	2.3 – 32.4: 1	Chapter 3
Bacterial load (molecular method)	55 - 2.8×10^{3} cell equivalents mL^{-1}	Chapter 3
Bacterial load (culture method)	0 - 3.1×10^{3} CFU mL^{-1}	Chapter 3
Primary bacterial functional potential	Amino acid metabolism Metabolism of other amino acids Metabolism of cofactors and vitamins	Chapters 3 and 5
Total haemocyte count	8.0×10^{5} - 1.3×10^{7} cells mL^{-1}	Chapter 3
Total granulocyte count	1.7×10^{5} - 4.9×10^{6} cells mL^{-1}	Chapter 3
Percentage of granulocytes	15 – 55 %	Chapter 5
Clotting time	18 to 57 s	Chapter 3

6.2 Gut microbiome

The core gut microbiome of *P. ornatus* consisted of Tenericutes and Proteobacteria (Chapter 4). Families *Pseudoalteromonadaceae* and *Vibrionaceae* were common in the *P. ornatus* gut. Unlike the haemolymph microbiome of healthy lobsters, vibrios were abundant in the lobster gut and likely to be involved in digestion and inhibiting competitor bacteria (Goulden et al., 2012a). Similar functions were also reported for *Pseudoalteromonas* spp. (Goulden et al., 2012a). The composition of the gut microbiome was shaped by gut region, where the hindgut exhibited higher bacterial diversity than the foregut and midgut (Chapter 4). Additionally, the hindgut microbiome was influenced by developmental stage with an increasing abundance of Tenericutes found with age. Members of BALOs were in low relative abundance in the gut (Chapter 5).

6.3 Comparison of gut and haemolymph microbiomes

The origin of naturally occurring haemolymph bacteria remains unclear, although the close association with water (Wang and Wang, 2015) and physical remodelling during moulting and metamorphosis are possibilities. However, bacteria are known to move from the digestive tract into the circulatory system and vice versa (Zhang et al., 2011). The core gut microbiome of healthy juvenile *P. ornatus* comprised phyla Tenericutes and Proteobacteria (Chapter 4) and this compares differently to the core microbiome of Proteobacteria and Bacteroidetes found in the haemolymph (Chapter 3). Bacteria found in both the gut and haemolymph with a mean relative abundance of > 1 % were *Flavobacteriaceae*, *Saprospiraceae*, *Rhodobacteraceae*, *Moraxellaceae*, *Pseudoalteromonadaceae* and *Vibrionaceae* (Table 6-2). These families could be transmitted from the gut into the haemolymph directly or indirectly.

Davis and Sizemore (1982) hypothesised that *Vibrio* spp. could enter the circulatory system of blue crab *C. sapidus* from the digestive tract via injury to the gut wall caused by migrating parasites. Additionally, the amount of *Vibrio* in the haemolymph of *C. sapidus* increased with warmer water temperatures (Davis and Sizemore, 1982) and handling injuries (Welsh and Sizemore, 1985). An example of direct transmission is the movement of bacterium *Borrelia burgdorferi* from the tick gut into the haemolymph when the tick feeds (Zhang et al., 2011), and indirect transmission may occur during remodelling of internal anatomy during moulting and metamorphosis (Zhukova et al., 2017). In insects, specialised cells known as bacteriocytes harbour bacterial endosymbionts which persist through various life stages via selective forces of the host immune system (Eleftherianos et al., 2013; Engel and Moran, 2013). However, it is likely that following impairment of circulatory immune responses that some community recruits originating from the gut may proliferate and cause systemic disease in lobsters.

It was found that host factors including tissue, organ region, and developmental stage influenced the microbiomes of lobsters (Chapter 4). Such host selection may be associated with targeted recruitment of functional bacteria and / or prevailing lobster ecophysiological conditions. The effect of developmental stage on haemolymph microbiome can be examined indirectly by comparing control lobsters in Chapters 3 and 5, where the proportion of Proteobacteria increased in the haemolymph of older juveniles. The high abundance of Proteobacteria in the circulatory system of *P. ornatus*, *C. gigas* (Lokmer and Wegner, 2015), and *C. sapidus* (Givens et al., 2013) suggests the importance of this phyla. On the contrary, the relative abundance of Proteobacteria in the hindgut of lobsters decreased with developmental stage. This shows that the microbial communities of digestive and circulatory systems are temporally dynamic yet generally distinct from one another.

Table 6-2. Bacterial families in the haemolymph and gut of *P. ornatus*.

Phylum	Class	Order	Family	Haemolymph (Chapters 3 & 5)			Gut (Chapter 4)		
				28C0d *n* = 6	28C6d n = 6	PBS *n* = 5	Fg13m *n* = 3	Mg13m *n* = 3	Hg13m *n* = 3
Actinobacteria	Actinobacteria	Actinomycetales	*Micrococcaceae*						+
Bacteroidetes	Bacteroidia	Bacteroidales	*Marinilabiaceae*					+	+
Bacteroidetes	Cytophagia	Cytophagales	*Flammeovirgaceae*			+			
Bacteroidetes	Flavobacteriia	Flavobacteriales	*Flavobacteriaceae*	+	+	+		+	
Bacteroidetes	Saprospirae	Saprospirales	*Chitinophagaceae*			+			
Bacteroidetes	Saprospirae	Saprospirales	*Saprospiraceae*	+	+	+		+	
Firmicutes	Bacilli	Bacillales	*Bacillaceae*					+	
Firmicutes	Clostridia	Clostridiales	*Acidaminobacteraceae*		+				
Firmicutes	Clostridia	Clostridiales	*Tissierellaceae*			+			
Lentisphaerae	Lentisphaeria	Lentisphaerales	*Lentisphaeraceae*		+				
Proteobacteria	Alphaproteobacteria	Kordiimonadales	*Kordiimonadaceae*		+				
Proteobacteria	Alphaproteobacteria	Rhizobiales	*Cohaesibacteraceae*	+	+				
Proteobacteria	Alphaproteobacteria	Rhizobiales	*Hyphomicrobiaceae*	+					
Proteobacteria	Alphaproteobacteria	Rhodobacterales	*Hyphomonadaceae*	+	+	+			
Proteobacteria	Alphaproteobacteria	Rhodobacterales	*Rhodobacteraceae*	+	+	+			+
Proteobacteria	Alphaproteobacteria	Sphingomonadales	*Sphingomonadaceae*			+			
Proteobacteria	Betaproteobacteria	Burkholderiales	*Burkholderiaceae*			+			
Proteobacteria	Betaproteobacteria	Burkholderiales	*Comamonadaceae*			+			
Proteobacteria	Deltaproteobacteria	Myxococcales	*Nannocystaceae*	+					
Proteobacteria	Gammaproteobacteria	Alteromonadales	*Alteromonadaceae*	+	+	+			
Proteobacteria	Gammaproteobacteria	Alteromonadales	*Colwelliaceae*			+			
Proteobacteria	Gammaproteobacteria	Alteromonadales	*Idiomarinaceae*			+			
Proteobacteria	Gammaproteobacteria	Pseudomonadales	*Moraxellaceae*		+				+
Proteobacteria	Gammaproteobacteria	Pseudomonadales	*Pseudomonadaceae*	+	+	+			
Proteobacteria	Gammaproteobacteria	Vibrionales	*Pseudoalteromonadaceae*			+	+	+	+
Proteobacteria	Gammaproteobacteria	Vibrionales	*Vibrionaceae*		+			+	+
Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	*Verrucomicrobiaceae*		+				
Tenericutes	Mollicutes	Mycoplasmatales	*Mycoplasmataceae*				+	+	+

+: family with $\geq$ 1 % mean relative abundance

6.4 Effect of temperature on cultured lobsters

As poikilothermic animals, lobsters cannot regulate body temperatures independent of environmental surrounds. It was shown (Chapter 3) that the upper thermal limit (34 °C) for *P. ornatus* is approximately 3 °C higher than the optimal growth range temperature (25 – 31 °C) (Jones, 2009). In comparison, a close relative of *P. ornatus*, the Caribbean spiny lobster *Panulirus argus* tolerates temperatures up to 35 °C (Reynolds and Casterlin, 1979). Although lobsters should be reared within their optimal temperature range, temperature is difficult to control particularly in sea cages. This is generally exacerbated by the positioning of sea cages in shallow coastal waters, yet these culture systems are also vulnerable to increased sea surface temperatures as predicted by climate change modelling (Norman-López et al., 2013). Therefore, farmers should monitor water temperature constantly and prevent mortality associated with heat stress by moving cages or animals.

Other consequences of thermal stress were discovered in our *P. ornatus* haemolymph studies (Chapter 3), where total granulocyte counts, and diversity, load and functional potential of the microbiome were significantly affected. The decrease in granulocytes could have reduced host immune response and defence against opportunistic bacteria. A stressed or compromised host and changes to prevailing ecophysiological conditions could also allow some bacteria to express different traits, such as virulence factors, converting them from commensals to pathogens. For example, *Pseudoalteromonas tunicata* have dual function traits whereby environmental conditions may induce it to change from a beneficial to a detrimental resident of algal hosts (Thomas et al., 2008). On the other hand, a shift in the functional potential of the haemolymph microbiome could drastically affect immune defences and titres and availability of nutrients (Chapter 3), leading to a range of systemic diseases. Similar to thermal stress, there were significant effects on bacterial diversity and load when the natural microbial composition was disrupted by experimental bacterial infection (Chapter 5).

6.5 Impacts of lobster microbiomic analyses

A sample from the circulatory system is the most accessible and practical choice in the diagnosis of systemic diseases. Given lobsters exhibit asymptomatic bacteraemia, haemolymph samples of both diseased and healthy animals are required to help diagnose bacterial diseases. Fortunately, haemolymph can be collected without killing lobsters as only a small volume is needed for microbiomic analyses. Moreover, molecular methods are preferred over culture-dependent methods because the former captures higher diversity and has greater resolving power when considering low bacterial loads of haemolymph. One can expect Proteobacteria and Bacteroidetes to be the predominant phyla and below 10^4 bacterial cells per milliliter of haemolymph in healthy *P. ornatus* (Chapters 3 and 5). These haemolymph bacterial parameters have the potential to be used in health monitoring programs and as a part of disease prevention measures.

Bacteria that can transfer from animals (including lobsters) to humans and cause disease are termed zoonotic. Zoonotic bacteria can be transferred to humans via ingestion or contact. As bacteria are present in the haemolymph and gut, there are transmission risks particularly when lobsters are served raw in sashimi. Potential zoonotic bacteria of *P. ornatus* haemolymph may include members of *Pantoea* spp. (Chapter 3) and specifically *Pantoea agglomerans* (Loch and Faisal, 2007) while the gut harbours a number of vibrios, where strains of *V. vulnificus* and *V. parahaemolyticus* are cited as zoonotic agents (Austin, 2010). It is recommended to disinfect lobster preparation areas, properly cook lobsters and clean open wounds to prevent zoonotic bacterial transmission.

6.6 Methods used for microbiomic studies in lobsters

The method used (culture-dependent or -independent) and volume of haemolymph were crucial factors for the detection of haemolymph and gut bacteria in this study. The culture-dependent approach required bacteria to be viable and conducive to growth on the provided medium, whereas the culture-independent method obtained DNA from viable, culturable but non-viable, and dead bacteria. This allows for the latter approach to capture a greater bacterial diversity and is more sensitive to lower bacterial loads when compared to culture-based plating methods (Chapters 2 and 3). Zhang et al. (2018) reported similar findings in the haemolymph of other crustaceans, mud crab *S. paramamosain* and Pacific white shrimp *L. vannamei* when the two methods were compared. A point of difference between culture-dependent and -independent methods was highlighted also in comparisons of gut bacteria (Chapters 2 and 4). All cultured phylotypes were identified in sequence libraries of culture-independent community analyses except a *Shimia* sp. isolated from the hindgut. This could indicate inter-individual variation.

The volume of haemolymph spread on each marine agar plate was limited to 100 µL. This sample volume can render a false negative result as bacterial load in the haemolymph can be low. However, when using the molecular method in this study, haemolymph amounting to as little as 1 % of animal wet weight and up to 500 µL was extracted with no apparent detrimental effect (Chapter 3). Nevertheless, animals < 3.3 g wet weight such as those used in Chapters 2 and 4 did not harbour sufficient volumes of haemolymph for molecular analyses if processed individually, and would require sample pooling.

Plasma was separated from the haemocytes and extracted to obtain bacterial DNA in this study (Chapters 3 and 5). The main advantage of using plasma was to minimise cross-reactions with host DNA and PCR inhibition. Cross-reactions occur due to co-amplification of mitochondrial 16S DNA (Sarver et al., 1998) of haemocytes (Hose et al., 1990) and bacterial 16S rDNA when 16S rRNA-based universal primers are used, leading to nonspecific PCR products. PCR inhibition of haemolymph was also reported in blue crab *C. sapidus* (Nagle et al., 2009) and oyster *C. virginica* (Kaufman et al., 2004). Moreover, PCR inhibitors may also come from haemolymph components such as lipids (Bligh and Scott, 1966), proteins (Johansson et al., 2000), and calcium ions (Paterson et al., 1997; Schrader et al., 2012), and anticoagulants such as EDTA (Montgomery-Fullerton et al., 2007). Nevertheless, it should be noted that bacteria detected in plasma are extracellular bacteria, and exclude bacteria that are within haemocytes.

There are several methods used to separate plasma from haemocytes in the haemolymph. A centrifugation step was employed in this study while Zhang et al. (2018) used filtration in another crustacean study. Zhang et al. (2018) filtered off the haemocytes using 5 µm pore size mesh filter membrane and the remaining plasma was filtered through 0.2 µm pore size membrane to collect bacterial cells. Although both methods remove haemocytes, there may still remain traces and fragments of haemocytes.

Decontamination is crucial when utilising highly sensitive molecular methods such as PCR given universal bacterial primers can detect trace amounts of bacteria. Dissection instruments were soaked in bleach and ethanol prior to flaming and sampling sites on lobsters were swabbed with ethanol during sampling. Bacterial contamination in the sampling and processing reagents was reduced by treatment with ultraviolet irradiation (Tamariz et al., 2006). Additionally, care was taken to minimise the introduction of bacteria, DNA and PCR amplicons via contact or air during processing by performing manipulations in biosafety cabinets, laminar flows, and in proximity to a flame. Moreover, extraction and no template controls were included in PCRs to allow any contaminating sequences to be subtracted from results of sequence libraries when present.

6.7 Future recommendations

It is envisioned that the technical underpinnings used in this study for microbiomic analyses can be applied to screen other spiny lobster species for asymptomatic bacteraemia. While haemolymph microbiomes of crustaceans are less studied when compared to gut microbiomes, knowledge of bacterial community dynamics (function, diversity, and load) in the haemolymph stands to challenge this convention in disease diagnostics. This approach abolishes the need to sacrifice an animal; although using faecal samples as a proxy for gut microbiomes could be an option should it be sampled in a manner that reduces contamination by waterborne microorganisms.

A number of extrinsic and intrinsic factors could significantly impact the dynamics of haemolymph communities. Comparison between wild animals and animals sourced from different culture systems and facilities may reveal that prevailing environmental conditions influence the survivorship of bacterial populations in the haemolymph. Further, as stated in Chapter 1, lobsters are more susceptible to bacterial invasion during pre- and post-moult stages. Hence, studying the changes of haemolymph microbiome in conjunction with haemocyte titres and activity at various phases of the moult cycle may help to answer crucial questions regarding sources and transmission routes of bacteria. Such transmission studies could require labelling of known resident bacteria of the haemolymph with fluorescent proteins.

Symbionts can potentially be developed into probiotics and it is recognised that autochthonous probionts are likely to be more functionally successful than probionts derived from other hosts (Hai et al., 2009). BALOs represent an alluring possibility as probionts or biocontrol agents and were found in *P. ornatus*, so their potential in the prevention and treatment of bacterial diseases should be explored further.

The functional taxonomy of lobster microbiomes should be explored in greater detail also. For example, KEGG predictions should be validated through functional assays given the limitations associated with predictive models that leverage from incomplete databases or those that contain inaccurate gene annotations. Alternatively, other computational methods that do not decouple taxonomic and functional information (e.g. FishTaco) and focus on which organism(s) contribute to functional changes of a community could be applied in future studies (Langille, 2018).

6.8 Conclusion

This study has provided the first comprehensive characterisation of the bacterial diversity and load in the haemolymph and gut of *P. ornatus* juveniles. Knowledge of the haemolymph microbiome will be foundational to future diagnoses of systemic diseases using non-destructive sampling methods. Consistently abundant haemolymph bacteria, such as family *Rhodobacteraceae* may be considered resident symbionts in healthy animals. Furthermore, bacteriolytic BALOs are also potential symbionts, likely to be involved in haemolymph population control and should be considered for biocontrol agents in disease management strategies. Overall, the haemolymph microbiome emerges as an integral part of the lobster hologenome (Desriac et al., 2014). The gut microbiome was influenced by gut region and developmental stage and a number of bacterial groups shared between the gut and haemolymph could suggest a yet to be defined transmission mechanism. This foundational study can be used to build health management strategies and reduce bacterial disease risks for the emerging lobster aquaculture industry.

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